Pituitary ovarian interaction in women with elevated early follicular phase FSH

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Pituitary ovarian interaction in women with elevated early follicular phase FSH

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door

Cornelia Hendrina de Koning

geboren te IJsselstein

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Aan mijn ouders

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1 Introduction and outline and aim of the thesis

Introduction

Day 3 serum Follicle Stimulating Hormone (FSH) has been shown to be prognostic of outcome in ovulation induction and assisted reproductive technology programs. The first report on basal FSH levels as a function of ovarian reserve in IVF date from 1988 (Muasher *et al.*, 1988). Many studies were performed since then and confirmed that elevated basal FSH levels are independent from age highly predictive of poor response in IVF (Scott *et al.*, 1989; Sharif *et al.*,1998; Toner *et al.*, 1991). Since day 3 FSH is believed to represent an indirect assessment of ovarian reserve, a measure of both follicular number and oocyte quality, and it is rising in normal ovarian aging while menses still occurs regularly (Sherman and Korenman, 1975) this thesis focusses on detailed endocrine description of this phenomenon.
The aim of this introduction is to review literature on the present knowledge of the physiology and pathophysiology of elevated FSH in the early follicular phase of women with regular menstrual cycles. We first discuss general aspects of female reproductive aging. Then elevation of FSH in relation to normal aging: endocrine aspects and menstrual cycle characteristics of female reproductive aging, will be discussed, and finally some other reasons for elevated FSH levels which are not related to diminished ovarian reserve will be discussed.

General aspects of female reproductive aging

Female reproductive aging is characterized by the decline in quantity and quality of the ovarian follicle pool. The follicle pool is established during early foetal life. In the 5th to 6th week of embryonic life, 1000-2000 primitive germ cells migrate from yolk sac to the developing ovaries (Crisp 1992). By mitosis, the number of germ cells and oogonia increases during the late embryonic and early fetal period (until the 5th to 6th month of gestation). By the second month of gestation, some 600,000 germ cells and oogonia are present in the fetal ovaries, and at the 5th month of fetal age there are approximately 7 x 10^6 potential egg cells. These oogonia serve the rest of the femal reproductive life span. From the 11th week of gestation oogonia are transformed into primary oocytes by starting the first meiotic division. The oocytes are arrested in the dictyate stage of the first meiotic division and are surrounded by one layer of granulosa cells and stored as primordial follicles. The process of atresia already starts during fetal life. At birth about 1 million follicles are left and 300.000 remain at menarche from which hundreds vanish every month thereafter. At menopause approximately 1000 oocytes still remain.
Faddy *et al.* (1992) developed a mathematical model that described the age-related decline in the number of small follicles in the human ovary. Their model demonstrated that the rate of follicle disappearance increased with age and more than doubled

when the numbers fell to 25.000 at 37.5 years of age. In vitro studies showed that at the same time, there is a quantitative decline in the remaining follicles, characterized by fewer granulosa cells per follicle, which have diminished function, indicated by less in vitro steroid and glycoprotein production, and also decreased mitosis and increased apoptosis (Seifer *et al.*, 1998).

Quantitative assessment of ovarian follicles

The decline in the number of primordial follicles with age has been studied by Block (1952, 1953) in his histological studies on post-mortem ovaries of women of varying ages. In these studies ovaries were fixed and slides were prepared of the ovarian tissue of complete ovaries. The total number of follicles per developmental stage in both ovaries was counted. Richardson (Richardson *et al.*, 1987) analysed one ovary of 17 women aged 45-55 years old by serial sectioning, and divided the cases in 3 groups by their menstrual history. The mean number of primordial follicles in the ovaries of women who were still menstruating regularly was 10-fold higher than that in perimenopausal women. Follicles were virtually absent in the postmenopausal ovaries. This group also found a rapid decline in follicle number in the peri-menopause and suggested that the hyper secretion of FSH stimulates a greater proportion of primordial follicles to enter the growing pool and thus accelerates the depletion of the primordial reserve.

Since the ovarian reserve depends on the number of primordial follicles, it seems reasonable to try to estimate the number of ovarian follicles directly by taking an ovarian biopsy. Attempts were made to quantify the number of primordial, primary and secondary follicles in small biopsies taken by diagnostic laparoscopy or open tubal surgery in 60 infertile women aged 19-45 years (mean 34.4 yrs), and there was a clear age dependent decline in follicular density (Lass *et al.*, 1997). Women over 35 years of age had only a third of the follicular density (number of follicles/ mm^3) compared with younger women. The number of follicles per unit of volume found in the biopsies was used to estimate the total and it was suggested that it could potentially be applied at the individual level. However, it was questioned that a biopsy for measurement of follicular density could accurately represent the density of the whole ovary (Lass, 2001).

Recently, several authors have shown that follicle density varied greatly in the cortex. Ovarian biopsies are therefore unreliable for estimation of an individual ovarian follicle content (Qu *et al.*, 2000; Schmidt *et al.*, 2003; Poirot *et al.*, 2002).

Endocrine aspects and menstrual cycle characteristics of female reproductive aging

Follicle Stimulating Hormone (FSH)

The first reports on elevated basal FSH levels date from 1975 by Sherman and Korenman. They published the first major study about serum gonadotrophin and sex steroid levels in women both early and late in their reproductive lives. Six cycles were studied in six women aged 46-51 years old, with regular menstrual cycles. The control group was made up of 10 subjects aged 18-30 years with regular menses. The authors noted a striking selective increase in the levels of serum FSH in the older regularly cycling women and significantly lower levels of serum oestradiol. LH and progesterone were not different from levels in the younger women. On the basis of these observations the authors postulated the existence of "an ovarian regulatory hormone, an inhibin, which would exert a negative feedback control over FSH secretion and which would be reduced in the years before menopause, consequent to a diminished number of follicles". Reyes *et al.* (1977) also reported a selective increase in the level of serum FSH in older regular cycling women. This was confirmed by Lee *et al.* (1988), who obtained daily samples for one cycle in 94 women with regular menses, ranging in age from 24-50 years. Serum FSH levels became progressively higher during the mid follicular and early postovulatory phase in women over the age of 40 years. LH levels were only elevated in the oldest age group (46- 50 years). Neither Reyes *et al.* (1977), nor Lee *et al.* (1988) found any significant age-related changes in the level of serum oestradiol or progesterone. Lenton *et al.* (1988) confirmed that FSH levels rose significantly in women over the age of 40 years when compared with younger women. A study by MacNaughton *et al.* (1992) also documented the selective rise in serum FSH during the early follicular phase of the menstrual cycle as a function of increasing age in regular cycling women. They also provided data on serum immunoreactive inhibin, which was lower in the older age groups in the follicular phase. This assay measured total immunoreactive inhibin, including inactive subunits and precursors. However, there was a significant negative relationship between immunoractive inhibin levels and increasing age.

Thus without any doubt we can say that rising levels of FSH are an irrefutable hormonal hallmark of reproductive aging.

Oestradiol

Oestradiol (E_2), secreted by granulosa cells in the ovary causes negative feed-back on pituitary gonadotrophin secretion via blocking hypothalamic GnRH secretion but also via direct effects on the pituitary. With regard to E_2 levels in the studies on regular cycling older women conflicting results were found. Some found lower levels (Sherman *et al.*, 1976; MacNaughton *et al.*, 1992; Lenton *et al.*, 1991), whereas

others described no changes (Santoro *et al.*, 2003; Reame *et al.*, 1996; Reyes *et al.*, 1977; Klein *et al.*, 1996a; 2000; 2002; Lee *et al.*, 1988) or an increase in oestradiol levels (Musey *et al.*, 1987; Klein *et al.*, 1996c; Santoro *et al.*, 1996; Kim *et al.*, 1997). Unfortunately, none of these authors described ultrasound findings to date multifollicular growth.
This inconsistency remains unclear. In our view explanations should be sought in high variability of quality and quantity of the oestrogen producing granulosa cells in relation to ovarian aging. In addition typical strong time frame shifts of the follicular phase hormonal events could have contributed.

Luteinizing hormone (LH)
Most studies but not all (Klein *et al.*, 1996; Mac Naughton *et al.*, 1992) show that LH levels also rise with age (Santoro *et al.*, 1996; Fitzgerald *et al.*, 1994; Lee *et al.*, 1988; Reame *et al.*, 1998). It seems that the increase of LH occurs later than that of FSH (Ahmed Ebbiary *et al.*, 1994). So far the increase of LH has been attributed to limitations of ovarian feedback. Possibly mechanisms underlying age related LH rising also contribute to earlier generation of the LH surge which in turn could explain the smaller maximum size of the follicle at the time of ovulation (Klein *et al.*, 1996a; van Zonneveld *et al.*, 2003; Fitzgerald *et al.*, 1994) and the consequent shortening of the follicular phase of the cycle and cycle length, a prominent clinical symptom of ovarian aging.

Progesterone
In most studies in older women with elevated FSH and a regular menstrual cycle the progesterone level is found to stay normal (Santoro *et al.*, 2003; Lee *et al.*, 1988; Reyes *et al.*, 1977; Klein et al, 1996a; Klein *et al.*, 1996c; Reame *et al.*, 1998; Welt *et al.*, 1999). However, one study reported lower progesterone levels in older women with elevated FSH levels (Muttukrishna *et al.* 2000) and longitudinal follow up over a period of 10 years demonstrated that progesterone secretion in the luteal phase does decline (Welt *et al.*, 2004). Lower progesterone levels in Fragile X premutation carriers with regular cycles and elevated FSH were also found (Welt *et al.*, 2004). In previously low responders in IVF, the progesterone levels seem to be elevated during the first stages of the follicular phase (Beckers *et al.*, 2002). This was interpreted as a result of remaining secretion from the corpus luteum of the previous cycle. Thus there are indications that over time progesterone levels become lower. This is probably resulting from more frequently occurring anovulatory cycles in older reproductive aged women.

Inhibins
Inhibins are members of the transforming growth factor-β superfamily. Inhibins are

heterodimeric molecules composed of a common α subunit and a β subunit, which confers specificity of action. Inhibin A consists of an α subunit with the β A subunit, and inhibin B consists of the same α subunit combined with the β B subunit. Inhibin A appears to be primarily secreted by the mature follicle and corpus luteum based on in situ hybridization and protein expression (Roberts *et al.*, 1993). The plasma concentrations of inhibin A in women also suggest the source of inhibin A to be the dominant follicle and corpus luteum (Groome *et al.*, 1996). Inhibin B appears to be a product of the smaller non-dominant antral follicles. Inhibin B is highest in the early follicular phase and falls approaching ovulation, and is low in the luteal phase (Groome et al, 1996). Both inhibin A and inhibin B directly suppress pituitary FSH secretion (Weiss *et al.*, 1993).

Older women with regular menstrual cycles show lower inhibin B concentrations (Santoro *et al.*, 1999; Santoro *et al.*, 2003, Klein *et al.*, 1996c; Welt *et al.*, 1999; Klein *et al.*, 2004; Danforth *et al.*, 1998, Muttukrishna *et al.*, 2000).

Older women show lower basal inhibin B levels than younger women but with normal inhibin B concentrations in the dominant follicle (Klein *et al.*, 1996c). It was concluded that the decreased basal inhibin B concentration probably reflects a decreased size of the antral follicle cohort. A decreased day 3 serum inhibin B precedes the early follicular phase rise of FSH in IVF patients with poor response (Seifer *et al.*, 1997).

Some studies also show inhibin A to be lower in older women (Santoro *et al.*, 1999; Welt *et al.*, 1999; Danforth *et al.*, 1998; Dudley *et al.*, 1998) but at a considerably later stage of the menopausal transition. Remarkably in the natural cycle of older (40-45 years) ovulatory women, inhibin A was elevated pre-ovulatory, while oestradiol levels were not different from younger women (Klein *et al.*, 1996c). This was postulated to be a consequence of stronger stimulation of the granulosa cells in older women by higher FSH concentrations. Higher inhibin A levels during the luteo-follicular transition (Klein *et al.*, 2004) and around day 6 of the follicular phase (Reame *et al.*, 1998) in older cycling women were described. Such early increases of inhibin A, a product of dominant follicles, may be another reflection of advanced follicle development in the same way as higher oestradiol levels in the early follicular phase are interpreted in this condition.

The follicular fluid content of inhibin A and inhibin B does not seem to differ between younger and older women. However, when in vitro stimulated luteinized granulosa cells from older women with pronounced elevated FSH levels were stimulated with FSH, lower inhibin A concentrations were found (Seifer *et al.*, 1996).

In conclusion, a decrease in inhibin B seems the most important and earliest factor that plays a role in the elevation of early follicular phase FSH, whereas lower inhibin A levels may govern FSH secretion in advanced stages of the reproductive aging process.

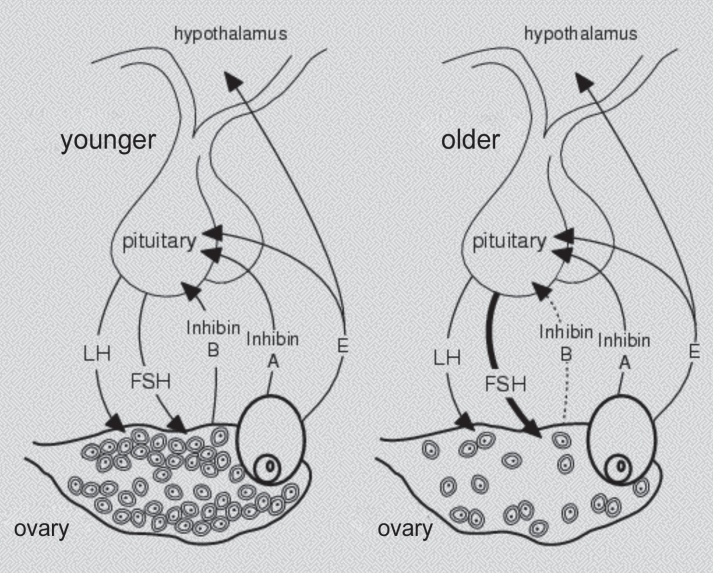

Figure 1. Concept of elevated FSH in reproductive aging

Length of follicular phase

A key feature of reproductive aging is shortening of the cycle length (Treolar *et al.*, 1967), which is largely attributable to shortening of the follicular phase. Many authors describe a shorter follicular phase length together with elevated early follicular phase FSH levels (Lenton *et al.*, 1984, Klein *et al.*, 1996a,b, Santoro *et al.*, 1996). In a population of 293 apparently ovulatory menstrual cycles from women aged between 18 and 39 years the distribution of follicular phase length, defined as the interval (in days) from the onset of menstruation up to, but not including, the day of the LH peak, there was a significant decrease (*P* less than 0.001) in follicular phase length with chronological age, from 14.2 days in women aged 18-24 years to 10.4 days in women aged 40-44 years (Lenton *et al.*, 1984). In another study perimenopausal women (47 years and older) showed a length of follicular phase 11 +/- 2 days vs. 14 +/- days in younger (19-38 yrs) women (*P* = 0.031) (Santoro *et al.*, 1996). The shortened follicular phase in older ovulatory women is due to shortening of the early section of the follicular phase, whereas the late follicular phase remains unchanged in length. Hormone profiles suggest that the growth of the dominant follicle is not accelerated (i.e. more rapid), but that its selection is advanced (i.e. earlier) (Klein *et al.*, 2002). Even after pituitary down-regulation with a GnRH agonist the follicular phase in older women was shorter compared with younger women. It was concluded that the shortening of the follicular phase is not dependent on hormonal influences

of the preceding luteal phase (Klein *et al.*, 2002; Messinis, 2006). Shorter cycles in older women compared to younger women could be a consequence of an earlier FSH rise in the preceding luteal phase causing protracted follicle growth and selection at an earlier stage (van Zonneveld *et al.*, 2003). Furthermore, shortening of the follicular phase could be explained if follicles ovulate at smaller diameter. This is indeed demonstrated in older women (Klein *et al.*, 1996a; van Zonneveld *et al.*, 2003; Fitzgerald *et al.*, 1994).

Contrary to these findings in older women recently Greb *et al.*(2005) described that in women with elevated FSH levels and a frequent single nucleotide polymorphism of the FSH receptor exchanging Asn for Ser at codon 680 homozygous (Ser680/Ser680), the interval from luteolysis (cycle 1) until ovulation (cycle 2) was significantly longer. They suggest that in these women the time needed for maturation of the FSH dependent cohort of follicles is increased in Ser680/Ser680-type women, because this FSH receptor is thought to be less sensitive to FSH. The underlying mechanism is different from older women with elevated FSH, where the follicle cohort is smaller. Women with elevated FSH levels and the Ser680/Ser680 FSH receptor variant basically have a normal follicle cohort.

So taken all together the well known shortening of the follicular phase with reproductive aging is likely to be the result of advanced start of follicle growth in combination with earlier ovulation.

Intercycle variability

Large intercycle variability of basal FSH predicts poor ovarian response upon stimulation in IVF (Scott *et al.*, 1990; Martin *et al.*, 1996). Women with a normal basal FSH show a small range of intercycle variation (Brown *et al.*, 1995). Patients with high intercycle variability of FSH responded poorly to gonadotrophin stimulation independent of their basal FSH concentration (Scott *et al.*, 1990). This was confirmed by others (Martin *et al.*, 1996) who showed that if the basal FSH at a single occasion was elevated, ovarian response to gonadotrophins was poor in every cycle. It was suggested that, in patients with high basal FSH, if the level returns to normal then the patient may have reasonable pregnancy rate (Lass *et al.*, 2000). So far, only one study examined this but found no better results (Abdalla *et al.* 2006). In this retrospective study however, samples used for the estimation of FSH were not collected in the beginning of the same IVF stimulation cycle from which outcome was measured.

Follicle growth velocity, maximal diameter of pre-ovulatory follicle and multiple follicle growth

As mentioned earlier shortening of the follicular phase is a key feature of reproductive aging. Increased growth velocity of the dominant follicle could be one of the

underlying mechanisms. So far, there are no reports of such accelerated growth. Literature reports on similar (Klein *et al.*, 1996a; van Zonneveld *et al.*, 2003) and even reduced growth velocity (Ahmed Ebbiary *et al.*, 1994).
The "normal" maximum size of a pre-ovulatory follicle is reported to be 17-25 mm (O'Herlihy *et al.*, 1980; Zegers-Hochschild *et al.*, 1984). All studies addressing follicular growth dynamics in ovarian aging report a reduction of the maximum diameter of the ovulatory follicle (Ahmed Ebbiary *et al.*, 1994; Klein *et al.*, 1996a; van Zonneveld *et al.*, 2003; Fitzgerald *et al.*, 1994).
Another feature of reproductive aging is the increased chance of natural dizygotic twinning for which multiple follicle growth is a prerequisite (Bulmer, 1970). Recently it was shown that older women show spontaneous multiple follicle growth up to 25% per cycle whereas this is only 5% in cycles of younger women (Beemsterboer *et al.*, 2006). Remarkably this was associated with higher FSH levels. More multiple follicle growth in older women was also described by others, but this was not statically different (Santoro *et al.*, 2003).
It remains unclear why follicles ovulate at a smaller diameter. Two hypothetical mechanisms are: 1) more multiple follicle growth causing higher oestrogen levels. The LH surge from the pituitary starts earlier as a result of this higher oestrogen levels, so the diameter of the follicles is smaller at the time of ovulation, and 2) decreased Gonadotrophin Surge inhibiting Factor (GnSIF) production making the pituitary more sensitive (see next paragraph) which could lead to an LH surge earlier with consequently a smaller diameter of the follicle.
Some years ago we described a patient that showed all features just mentioned (Lambalk *et al.*, 1998). She was 36 years old and had FSH levels of 18 IU/l in a previous cycle. We monitored in detail the endocrine and follicle dynamics in the following cycle. The figure shows the luteo-follicular transition details:

1. Follicles started to grow during the luteal phase of the preceding cycle.
2. There was ongoing multiple follicle growth.
3. Growth velocity was normal (± 2 mm/day).
4. Maximum diameter of the follicles was 16-17 mm and slightly smaller then normal.
5. Ovulation occurred during menstruation (note shifts in BBT and progesterone levels).

GnSIF/GnSAF
Gonadotrophin surge inhibiting factor/gonadotrophin surge-attenuating factor, a non-steroid hormone, is secreted by granulosa cells of many species including the human, and keeps the pituitary in a low state of responsiveness to GnRH (de Koning 1995, Fowler and Templeton 1996, Fowler *et al.*, 2003). Unfortunately despite its clear

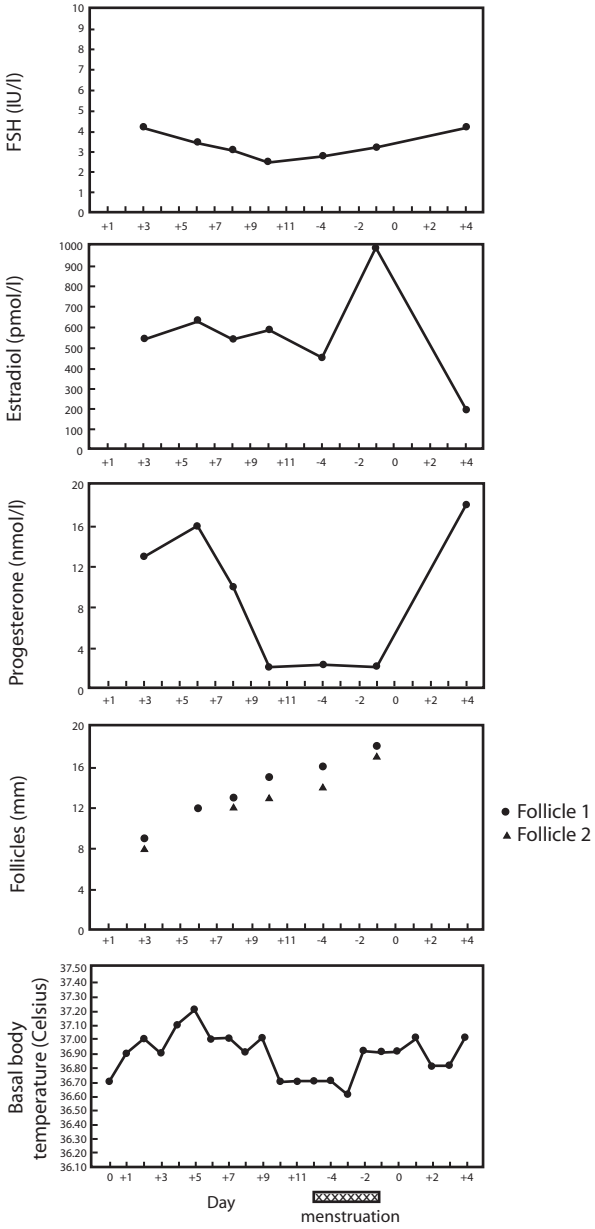

Figure 2. Patterns of serum FSH, oestradiol and progesterone (upper 3 panels) and follicle growth and basal body temperature (lower 2 panels) of a 36 years old patient who had an FSH concentration of 18 IU/l in a previous cycle, showing typical ovulatory changes as rise in basal body temperature and progesterone production during menstruation while progressive multiple follicle growth was observed during the luteal phase of the preceding cycle. Please note absence of elevated FSH levels during this particular period of monitoring.

biological presence so far attempts to identify this factor have been unsuccessful. Changes in GnSIF activity are thought to play a role in the generation of the midcycle LH surge. In the natural menstrual cycle the pituitary sensitivity to GnRH increases in the late follicular phase, i.e. during the pre-ovulatory period (Messinis *et al.*, 1994, 1998). Small follicles contain high concentrations of GnSIF bioactivity, whereas larger follicles contain low GnSIF concentrations (Fowler *et al.*, 2001). So far, only a bioassay is available to measure GnSIF serum concentrations (Fowler *et al.*, 2001). Nevertheless, a decreased GnSIF bioactivity in the spontaneous cycle in a group of women with poor response in IVF was found (Martinez *et al.*, 2002).
Recently in a study with post-menopausal women with exogenous oestrogen and progesterone substitution, the pituitary sensitivity to GnRH was tested with GnRH challenge tests (Dafopoulos *et al.*, 2004). Based on these studies it was concluded that during the early and midfollicular phase the ovaries produce GnSIF that antagonizes the pituitary-sensitizing effect of E_2 to GnRH. This is in line with the finding that higher bioactivity of GnSIF was found in the circulation of normal cycling women in the early and midfollicular phase compared to the late follicular phase (Martinez *et al.*, 2002).
Lower rates of GnSIF secretion could thus to some extent explain the higher levels of LH and FSH as seen in women with limited ovarian reserve.

Activin A
Activins, like inhibins, are members of the transforming growth factor-β superfamily and are present in many tissues and also found in follicular fluid. Activins are homodimers or heterodimers consisting of only β-subunits. The homodimers $\beta_A\beta_A$ and $\beta_B\beta_B$ are called activin A and activin B, respectively. Activin AB is a heterodimer consisting of β_A- and β_B- subunits. Activins are capable of selectively stimulating FSH secretion by pituitary cells.
Two studies (Reame *et al.*, 1998; Santoro *et al.*, 1999) reported an increase in serum Activin A levels in older ovulatory women and suggested that an increase in Activin A could be a factor in the monotropic FSH rise. On the other hand in subsequent studies no difference in activin A levels in older (40-45 yr) ovulatory women was found (Klein *et al.*, 2004). Activin A has been shown to increase with age in both men and women but does not correlate with FSH levels (Hurwitz and Santoro, 2004; Baccarelli *et al.*, 2001, Loria *et al.*, 1998). Furthermore, mRNA for activin subunits is expressed in a number of extragonadal tissues (Tuuri *et al.*, 1994), is present predominantly in a bound (and therefore inactive) form in the circulation (McConnell *et al.*, 1998), and does not vary across the menstrual cycle despite marked variability in FSH levels. Although our current understanding of activin physiology is limited by the lack of available assays for other activin forms (e.g. activin B, activin AB), to date there is little evidence to support an endocrine role for activin in FSH regulation.

Anti-Müllerian hormone

Anti-Müllerian hormone (AMH) is another member of the transforming growth factor-β family of growth and differentiation factors. AMH, also known as Müllerian Inhibiting Substance (MIS), plays a role during male sex differentiation, where it signals the regression of Müllerian ducts. In the postnatal female AMH, which is produced in the granulosa cells, regulates growth and development of ovarian follicles. In women AMH is expressed in primary follicles immediately after they have started to grow i.e. after recruitment, whereas its expression ceases in follicles that have been selected for dominance. This expression pattern is consistent with a role of AMH at two major regulatory checkpoints of follicular development:1) the entrance of the resting primordial follicles into the growing pool and 2) the exit of follicles either to ovulation or death (atresia). From AMH null-mice two major functions of AMH have been learned: 1. AMH inhibits primordial follicle recruitment and 2. AMH inhibits the growth stimulatory effects of FSH. The number of small antral follicles is related to the size of the primordial follicle pool (Gougeon, 1984). With the decrease in the number of the antral follicles with age, AMH production appears to become diminished (van Rooij *et al.* 2004; van Rooij *et al.*, 2005).

The usefulness of serum AMH as a marker of ovarian reserve was tested in a group of 41 normal ovulatory women at two times with an average interval of 2.6 ± 1.7 years. AMH serum levels significantly decreased between the two samples and a negative correlation was found between age and AMH levels. Furthermore, AMH showed a strong correlation (r=0.66 and r=0.71 respectively, for first and second sample) with the antral follicle count (AFC) (de Vet *et al.*, 2002).

Several studies have shown that serum AMH levels are strongly correlated with the antral follicle count, the number of follicles retrieved in IVF, age, inhibin B and FSH (van Rooij, *et al.*, 2005; Seifer *et al.*, 2002; Fanchin *et al.*, 2003; Visser *et al.*, 2006; La Marca *et al.*, 2006; Ficicioglu *et al.*, 2006).

Recent studies suggest that AMH levels do not vary much throughout the menstrual cycle (Cook *et al.*, 2000; La Marca *et al.*, 2002; Hehenkamp *et al.*, 2006), and it is constant in the same women from one cycle to the next (Fanchin *et al.*, 2005) Thus, serum AMH seems to be an easy to obtain marker of ovarian reserve.

Other factors related to diminished oocyte reserve

Genetic factors determine for a major part the onset of natural menopause. Women with a family history of early menopause are a high-risk group for experiencing early menopause (de Bruin *et al.*, 2001; te Velde *et al.*, 2002). Galactosaemia is an inherited inborn error of the galactose assimilation pathway which results in accelerated ovarian apoptosis and ovarian failure (Forges *et al.*, 2006). Premutation carriers of Fragile X syndrome (FMR1 gene) have a relative risk of 21 % of premature ovarian failure (POF) (Sherman, 2000). The underlying pathophysiology of POF caused by

the premutation allele is not understood.
Other, acquired factors include: chemotherapy, radiotherapy, pelvic surgery (Lass *et al.*, 1998; Tulandi *et al.*, 2002), pelvic infections or tubal disease (Keay *et al.*, 1998; Sharara, 1998), severe endometriosis (Barnhart *et al.*, 2002), and smoking (Augood *et al.*, 1998). Moderate-to-heavy smokers (14 or more daily cigarettes) experience menopause 2.8 years earlier than never smokers (Kinney *et al.*, 2006).
Recently a higher prevalence of premature ovarian failure was described in monozygotic and dizygotic twins (Gosden *et al.*, 2007). An explanation for the latter finding is probably for dizygotic twins an earlier menopause in their mothers, related to higher FSH levels and as a consequence multiple follicle growth and ovulation. By genetic inheritance the daughters will also experience earlier menopause. For monozygotic twinning it is more difficult to find a plausible explanation for an earlier menopause. In families with a history of dizygotic twinning, variants in the growth differentiation factor-9 (GDF9) gene were found more often than in controls (Palmer *et al.*, 2006). GDF9 is an oocyte-derived growth factor essential for follicle growth. Recent papers describe rare variants in both GDF9 and BMP15 contributing to POF (Di Pasquale *et al.*, 2006; Laissue *et al.*, 2006) A relationship between twinning and POF may be found in GDF9 and BMP15 gene variants.

Hypothalamic-pituitary aspects of aging
Studies on episodic LH secretion and pituitary GnRH responsiveness in women after menopause have shown that the activity of the hypothalamic pulse generator declines dependently on calendar age, probably reflecting age dependent neuronal loss, whereas pituitary responsiveness declined in relation to the duration of menopause, probably a reflection of oestrogen deprivation (Lambalk *et al.*, 1997).
The pituitary gland has also been studied in relation to the elevation of FSH in older premenopausal women by several investigators, with conflicting results. LH pulse studies were performed by Klein (Klein *et al.*, 1996d) and Wilshire (Wilshire *et al.*, 1995) who found no differences in LH pulse frequency and amplitude in older women whereas others described an increased LH pulse amplitude in the late luteal phase in older cycling women (Reame *et al.*, 1996). Some authors suggest that the monotropic rise of FSH in the perimenopause results from a lower GnRH pulse frequency (Reame *et al.*, 1996). In the past studies in hypothalamic lesioned ovariectomized monkeys showed that lower GnRH pulse frequencies selectively increase FSH secretion (Belchetz *et al.*, 1978) However, such mechanism could not be shown in human with intact ovarian feedback activity (Lambalk *et al.*, 1997). So far, no studies substantiated a significant lowering of the LH pulse activity as a reflection of hypothalamic GnRH pulse activity in perimenopausal women.
Other studies evaluated the response to GnRH test doses. In peri-menopausal women with irregular cycles, the FSH and LH response to GnRH (100 μg) were elevated

in the early and mid follicular phase (Schmidt *et al.*, 1996). However, Fujimoto (Fujimoto *et al.*, 1996) found no differences in LH and FSH response after 30 minutes with a lower GnRH test dose (25 μg). The FSH peak in older women was found to be higher compared with younger women, indicating a more pronounced pituitary FSH secretion, while steroids and inhibins were not different between the groups (Klein *et al.* (1996a).

In conclusion, there are no strong indications that typical endocrine changes of the reproductive axis in relation to the perimenopausal transition have a hypothalamic/pituitary origin.

Factors related to elevated FSH, but *not* to diminished ovarian reserve

FSH receptor polymorphisms

The FSH receptor belongs to the family of the G protein-coupled receptors inducing signal transduction by protein kinase A/cAMP pathway (Simoni *et al.*, 1997). Apart from rare mutations, two common single nucleotide polymorphisms (SNP) were identified in exon 10 of the FSH receptor gene at nucleotide position 919 and 2039, respectively. The first SNP is located in the extracellular domain at codon position 307, which can be occupied by either alanine (Ala) or threonine (Thr). The second SNP located in the intracellular domain at codon position 680 changes an asparagine (Asn) to serine (Ser) (N680S).

In studies in controlled ovarian hyperstimulation cycles evidence was found that the amino acid transition Asn680 to Ser680 (N680S) results in subtle differences in receptor function as reflected by higher basal FSH levels in the early follicular phase and/or the number of ampoules required for ovarian stimulation (Perez-Mayorga *et al.*, 2000; Simoni *et al.*, 2002; de Castro *et al.*, 2003). These findings suggest that this receptor variant is less sensitive to FSH and that higher endogenous FSH levels may represent a natural compensation which is needed to enable normal follicle growth.

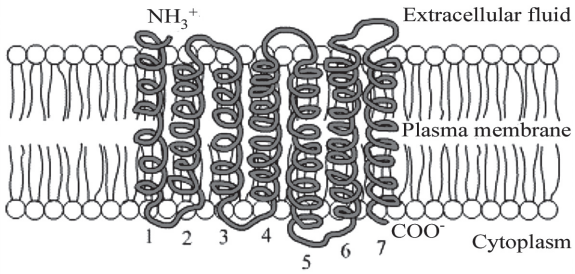

Figure 3. The seven transmembrane α-helix structure of a G protein-coupled receptor such as FSHR

In a group of normogonadotropic anovulatory women, the homozygous N680S variant was found to be more prevalent with higher basal FSH levels (Laven *et al.*, 2003). In these situations not a limited ovarian reserve, but a higher FSH threshold is responsible for the elevated FSH levels in the early follicular phase.

Interferences in immunometric assays for FSH

Elevated serum FSH levels in women with regular menstrual cycles can also be caused by technical laboratory errors. In most laboratories, FSH concentrations in serum are measured by two-side immunometric assays. The presence of heterophilic antibodies that interfere with the immunoassay, gives falsely elevated FSH levels. Falsely elevated serum FSH concentrations measured with immunoassays have been reported in women with a history of vaccination with inactivated bacteria cultured in rabbit tissue (Padova *et al.*, 1991) and in a patient who had worked with rabbit serum (Cahill *et al.*, 1992). If elevated FSH levels in regular cycling women are not trusted, it is recommended to verify the FSH values by serial dilution and by precipitation with polyethylene glycol (PEG) to rule out interference in the assay.

Familial dizygotic twinning

Familial dizygotic twinning is associated with elevated early follicular phase FSH concentrations in the normal ovulatory cycle (Lambalk *et al.*, 1998; Thomas *et al.*, 1998; Martin *et al.*, 1991). Multiple follicle growth and subsequent multiple ovulation seems to be a logical explanation. In a prospective clinical study of the pulsatile secretion of FSH and response to GnRH on cycle day 3, in mothers who had had dizygotic twin pregnancy, mean FSH concentrations and FSH pulse frequency were significantly elevated, while no differences in oestradiol, inhibin A and inhibin B were found (Lambalk *et al.*, 1998). It was concluded that twin mothers have hyperstimulation by endogenous FSH caused by neuroendocrine, hypothalamic or pituitary mechanisms.

In a large sib pair study of mothers with dizygotic twins, a negative logarithm of the odds (LOD) score for markers at the locus of the FSH receptor was found, which makes involvement of the FSH receptor in this condition unlikely (Montgomery *et al.*, 2001; Lambalk *et al.*, 2001).

Gonadotroph cell adenomas of the pituitary

A rare cause of elevated FSH levels is the presence of gonadotroph cell adenomas of the pituitary secreting Follicle-Stimulating Hormone. The most common hormonal characteristic of gonadotroph cell adenoma is hypersecretion of FSH, which is often accompanied by hypersecretion of FSH beta and alpha subunit (Snyder, 1985). Most gonadotroph cell adenomas are treated by transsphenoidal surgery and radiation.

Fourteen cases of spontaneous ovarian hyperstimulation caused by gonadotrophin

producing pituitary adenoma have been reported in literature (Kihara *et al.*, 2006). These cases show elevated oestradiol levels, low LH levels and often irregular menstrual cycles and visual impairment.

Autoimmune disease

In women with Premature Ovarian Failure (POF) autoimmune abnormalities are a well known phenomenon. In a population of POF patients attending a reproductive endocrinology unit the incidence was 1 % (Conway, 1997). Most patients show specific antibodies against steroid-producing cells of the ovary and the adrenal gland. In women with regular menstrual cycles and elevated early follicular phase FSH levels a higher incidence of ovarian antibodies in serum was found (Cameron *et al.*, 1988; Ahmed Ebbiary *et al.*, 1994). However, in the study in which we participated, no difference was found in autoantibodies between controls and women with elevated FSH levels (van Kasteren *et al.*, 2000).

Treatment of women with elevated FSH and ovarian antibodies with corticosteroids could be a good option (Rabinowe *et al.*, 1986; Corenblum *et al.*, 1993) if they are amenorrhoic or oligomenorrhoeic. However, in a randomized placebo-controlled study with idiopathic POF patients, corticosteroids did not influence ovarian responsiveness to gonadotrophins (van Kasteren *et al.*, 1999). Treatment with corticosteroids and gonadotrophins in IVF stimulation protocols in patients with ovarian antibodies result in a high number of retrieved follicles (Forges *et al.*, 2006; Tozer *et al.*, 2004). Hoek *et al.* (1997) concluded that POF in association with adrenal autoimmunity and/or Addison's disease is indeed an autoimmune disease and for these patients immunomodulating therapies may be successful.

Outline and aim of the thesis

In this introduction the concepts of elevated FSH in regular menstrual cycles were outlined and discussed. Most insight with regard to understanding the endocrinology of elevated basal FSH came from studies in older women whereas an infertility population is relatively younger. We therefore designed an observational cohort study in a group of relatively young regular cycling women with elevated early follicular phase FSH levels and controls. For proper use of basal FSH in clinical settings we considered it necessary to have better insight in the endocrinology behind elevated FSH secretion in natural menstrual cycles. It is well known that basal FSH levels can vary in patients between cycles. The study group was therefore divided into women that had repeatedly elevated FSH and women that had variably elevated FSH. The detailed hormone analysis is described in chapter 2.

Gonadotrophin secretion by the pituitary is the net result of stimulating brain factors and inhibiting gonadal factors. In order to understand the mechanisms that

contribute to alteration of these episodically secreted hormones we felt that is was necessary to study pulsatile gonadotrophin secretion and pituitary response to GnRH (chapter 3).
Many studies with regard to the ovarian reserve issue refer to the "gold standard" as it should be: a biopsy of the ovary to find out the actual number of follicles left in the ovary. In chapter 4 we describe a study which was performed to tackle the problem of estimation of the number of ovarian follicles in the ovary by taking biopsies. We performed a histological study to find out if and how a representative biopsy could be taken.
A higher FSH threshold for follicular growth could also be a cause of elevated FSH levels in women with regular menstrual cycles and elevated early follicular phase FSH levels. Therefore we estimated the threshold for FSH according to a previously developed and validated method in women with elevated FSH and compared this with controls (chapter 5).
Heterophilic antibodies interfering in the immunometric assay for FSH are known to be potentially responsible for the elevated FSH levels found. Chapter 6 describes three cases that were part of our study population, in which elevated day 3 FSH had nothing to do with diminished ovarian reserve.
In recent years a frequent single nucleotide polymorphism of the FSH receptor gene exchanging Asn for Ser was found and this receptor polymorphism is likely to have another sensitivity for FSH. The hypothesis is that a less sensitive FSH receptor needs more FSH to stimulate the receptor. This can thus be another cause for elevated FSH levels not related to ovarian reserve. Accordingly, we evaluated the FSH receptor genotype distribution in subfertile women with elevated FSH in comparison to controls (chapter 7).
Finally, the results and conclusions of the studies are discussed in the General Discussion.

The general aim of the thesis was to unravel mechanisms behind elevated FSH. We specifically aimed:

1. to describe in detail the reproductive endocrinology of women with regular menstrual cycles and elevated day 3 FSH levels.
2. to evaluate the neuroendocrine mechanism responsible for elevated FSH, in women with regular menstrual cycles.
3. to assess the issue of ovarian reserve estimation by taking ovarian biopsies.
4. to evaluate the ovarian FSH threshold in women with elevated FSH and a regular menstrual cycle.
5. to evaluate the distribution of FSH receptor variants in women with elevated basal FSH levels and a regular menstrual cycle.

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2 The endocrine and follicular growth dynamics throughout the menstrual cycle in women with consistently or variably elevated early follicular phase FSH compared to controls

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Abstract

Background: Elevated early follicular phase FSH is frequently observed in subfertile patients. We studied the complete endocrine cycle profile of subfertile young women with elevated basal FSH compared to controls.

Methods: Daily bloodsampling and ultrasound monitoring in the follicular phase was performed in 22 patients with elevated basal FSH levels in screening, and 16 controls during one menstrual cycle and 5 days of the next cycle.

Results: 11 patients showed elevated basal FSH levels in the study cycle ("High, High"; H,H group) whereas 11 had normalized basal FSH levels ("High, Low"; H,L group). AMH was lower in both groups. In the H,H group, FSH was higher in all phases of the cycle and both inhibin A and inhibin B were lower during the early follicular phase. In the H,L group FSH was also higher in the early follicular phase and late luteal phase, and inhibin A was higher in the peri-ovulatory phase."Normalization" of day 3 FSH in women with previously elevated FSH was associated with normalization of inhibin B levels in the preceding luteal phase.

Conclusion: The endocrine cycle profile in subfertile patients with consistently elevated basal FSH resembles that of published data from older women. Normalization of FSH in association with normal inhibin B, suggests a temporary increase of the available cohort.

Introduction

Elevated early follicular phase serum levels of FSH have been used in recent years to counsel patients in infertility treatment, because this is associated with poor response in ovarian hyperstimulation and subsequent low pregnancy rates (Scott and Hofmann, 1995). This monotropic rise of FSH levels in the early follicular phase has been described by a number of authors (Sherman *et al.*, 1976; Reyes *et al.*, 1977; Lee *et al.*, 1988) in ageing ovulatory women. More recent investigations have shown that in older women in the early follicular phase serum inhibin B levels decrease (Klein *et al.*, 1996, 2004; Danforth *et al.*, 1998; Welt *et al.*, 1999; Muttukrishna *et al.*, 2000) and also in younger women with elevated FSH levels (de Koning *et al.*, 2000). It is assumed that decreased negative feedback from the diminishing number of pre-antral and early antral follicles plays a major role with regard to this FSH rise. Hypothalamic causes for higher FSH levels, as in mothers of dizygotic twins with more frequent GnRH pulses (Lambalk *et al.*, 1998a), could also play a role, but in an earlier study we investigated the FSH pulse rate in women with elevated FSH levels in the early follicular phase and found that this is not the case (de Koning *et al.*, 2000). In the latter study higher FSH and LH pulse amplitude and response to GnRH in a group of women with elevated day 3 FSH levels were found in combination with lower inhibin A and inhibin B levels. Apart from increased early follicular FSH levels, anti-Müllerian hormone (AMH) has been suggested to be an early marker of female reproductive ageing (de Vet *et al.*, 2002; van Rooij *et al.*, 2002; Fanchin *et al.*, 2003). AMH is produced by primary, secondary and early antral follicles and its concentrations do not vary throughout the menstrual cycle (Cook *et al.*, 2000; La Marca *et al.*, 2004; Hehenkamp *et al.*, 2006).

Often the elevated early follicular phase FSH is not detected in every cycle of subfertile women. Many patients occasionally show normal levels (Jain *et al.*, 2003; Brown *et al.*, 1995). So far, no detailed knowledge is available about the cycle pattern of reproductive hormones in patients that show a temporary normalization of basal FSH. This makes understanding of this phenomenon and its possible consequences difficult. Some authors have suggested that IVF treatment in such an occasional cycle may lead to better results than in cycles with increased FSH levels (Lass *et al.*, 2000), although others indicate no differences in response and pregnancy outcome (Scott *et al.*, 1990; Abdalla and Thum 2006).

All reports detailing the menstrual cycle pattern of the reproductive hormones in relation to the issue of limited ovarian reserve commonly compare older versus younger patients rather than making a comparison between women with or without elevated FSH irrespective of age. Therefore, the aim of the present study was to evaluate in detail the endocrine hormonal profile and follicle development of relatively younger women with elevated day 3 FSH compared with women around

the same age showing normal FSH as it is not evident that the endocrine profile in these women resembles that in published data from older women. We explicitly distinguished between patients with persistently elevated FSH and those showing a temporary normal value. The study design which incorporated 2 menstrual periods allowed us to closely evaluate hormonal determinants of the luteo- follicular transition that associate with early follicular phase gonadotropin levels.

Material and methods

Subjects

Patients, referred to our infertility clinic, were all screened on day 3 of the menstrual cycle. All patients with day 3 FSH values of ≥ 10 IU/l were asked to participate in the study. In our IVF clinic, patients with basal FSH values ≥ 10 IU/l show very poor outcome with respect to the number of oocytes retrieved and pregnancy rate. Controls were either patients referred to our clinic for reversal of tubal ligation or volunteers recruited by advertisement, with cycle day 3 values of < 10 IU/l ("Low, Low" (L,L) group) . All women had regular menstrual cycles (21-35 days), normal endocrine screen, no current medical illness, no current use of oral contraceptives, no pregnancy or breastfeeding in the past 6 months. The study patients were divided into two groups: 11 of the 22 patients had again elevated FSH concentrations on day 3 of the study cycle, this was the "High, High" (H,H) group, and 11 patients had FSH concentrations < 10 IU/l on day 3 of the study cycle ("High, Low" (H,L) group). All women were ovulatory (as determined by serial ultrasound monitoring and elevated serum progesterone levels in the luteal phase above 15 nmol/l) in the study cycle and cycles were 21-35 days in duration.

Study design

Daily bloodsamples were obtained, starting from day 1 of menstruation until day 5 after the beginning of the next menstruation. Serum was stored at –20 ^{0} until processing. Transvaginal ultrasound was performed on day 3, day 8, and every other day until a follicle of 14 mm diameter was seen. Daily ultrasound was performed from then on. Follicular collapse or an increase in echogenicity of the large follicle were considered to represent ovulation. In the luteal phase daily bloodsampling was continued. FSH, LH, estradiol, inhibin B, inhibin A and progesterone was measured in all samples. Anti-Müllerian Hormone (AMH) was measured on day 3 of two consecutive cycles.

The study was performed in accordance with current guidelines on good practice in clinical research and the Declaration of Helsinki. The study was approved by the Institutional Review Board, and written informed consent was obtained from all participants.

Hormone measurements

LH and FSH were measured in duplicate by commercially available immunometric assays (Amerlite; Amersham, Bucks, UK). The lower limit of detection was 0.3 IU/l for LH and 0.5 IU/l for FSH. The assays were calibrated against the 1st International Reference Preparation (IRP) 68/40 and the 2nd IRP 78/549 for LH and FSH respectively. Of each individual, all samples were analysed in the same run for each hormone. The inter- and intra-assay coefficients of variation (CV) were < 9 and 5 % for LH and FSH.

Inhibin A and inhibin B were measured in duplicate by ultra sensitive two-site enzyme immunoassays (Serotec, Oxford, UK). The lower limit of detection was 3 pg/ml for inhibin A and 15 pg/ml for inhibin B. Estradiol was measured by radioimmunoassay (Sorin Biomedical, Sallugia, Italy) with a lower limit of detection of 18 pmol/l and an inter-assay CV of < 11%. For progesterone a commercially available competitive immunoassay (Delfia, Wallac Turku, Finland) was used with an intra-assay CV of 4-8 % and inter-assay CV of 7-13 %.

An ultra-sensitive immuno enzymometric assay kit (Diagnostic Systems Laboratories, Webster, TX) was used for estimation of AMH (Al - Qahtani *et al.*, 2005). The limit of detection (defined as blank + 3SD of blank) was 0.08 μg/L. Intra-and interassay coefficients of variation were <5%.

Transvaginal ultrasound

All transvaginal ultrasound measurements were performed by the same observer (C.H.dK) using a 7.5 MHz transvaginal probe (Aloka SSD-1700). Only follicles > 10 mm were measured by taking the mean of three perpendicular measurements. The two layers of the endometrium together were measured as endometrial thickness.

Statistical analysis

Between-groups baseline data were compared using weighted least squares regression models and chi-squared tests. Weighted least squares analyses were used since the variance within groups varied significantly. To compare hormone levels between groups, the day of LH peak was defined as day 0. The menstrual cycle was divided into 6 phases: early follicular (15 to 11 days before LH peak), midfollicular (10 to 6 days before LH peak), late follicular phase (5 to 1 days before LH peak), and early luteal (1 to 5 days after LH peak), midluteal (days 6 to 10) and late luteal (days 11 to 15) phases. Days outside this range were ignored due to paucity of observations. For each phase, the average hormone level per patient was calculated using that patient's daily values. Groups were again compared using weighted least squares regression since within group variance differed signficantly. Hormone levels on the day of the LH peak were also compared using ANOVA. Follicle growth and maximal follicle diameter before ovulation was calculated

Table 1. Patient characteristics. Values are presented as mean (SD)

	H,H group (*n*=11)	H,L group (*n*=11)	L,L group (*n*=16)	
Age (y)	35.6 (4.5)	35.6 (2.6)	34.4 (3.7)	n.s
BMI	21.4 (1.6)	22.4 (2.2)	24.1 (4.5)	n.s.
Day 3 FSH screening (IU/l)	14.0 (4.3)	15.5 (6.8)	4.5 (1.0)	H,H=H,L<L,L; *P*<0.001
Packyears	7.7 (6.4)	6.5 (11.1)	3.9 (5.7)	n.s.
Duration infertility (y)	3.4 (3.4)	2.9 (1.5)	n.a.	n.s.
Age menarche (y)	12.6 (0.7)	12.5 (1.2)	12.25 (1.2)	n.s.
Age menopause mother (y)	47.5	50.7	53	H,H<H,L=L,L; *P*<0.05

H,H= High, High group: elevated basal FSH in screening and in the study cycle
H,L= High, Low group: elevated basal FSH in screening and < 10 IU/l in study cycle
L,L= Low, Low group (controls)

Table 2. Day 3 values 1st cycle. Values are presented as mean (SD).

	H,H group (*n*=11)	H,L group (*n*=11)	L,L group (*n*=16)	
AMH (µg/l)	0.40 (0.35)	0.50 (0.29)	3.94 (3.24)	H,H=H,L<L,L; *P*<0.0001
FSH (IU/l)	17.2 (6.5)	6.2 (1.4)	4.4 (1.0)	H,H>H,L>L,L; *P*<0.001
LH (IU/l)	6.1 (1.1)	3.1 (1.5)	2.9 (1.1)	H,H>H,L=L,L; *P*<0.01
E_2 (pmol/l)	100 (17)	165 (85.2)	110 (21)	n.s.
Progesterone (nmol/l)	1.9 (0.8)	1.9 (0.9)	1.5 (0.8)	n.s.
Inhibin A (ng/l)	4.1 (1.8)	10.4 (1.9)	7.3 (2.9)	H,H<H,L=L,L; *P*<0.005
Inhibin B (ng/l)	53.1 (50)	97.9 (0.9)	103.3 (42.1)	H,H<H,L=L,L; *P*<0.05

H,H= High, High group: elevated basal FSH in screening and in the study cycle
H,L= High, Low group: elevated basal FSH in screening and < 10 IU/l in study cycle
L,L= Low, Low group (controls)

Table 3. All values are mean and SD

	H,H group (*n*=11)	H,L group (*n*=11)	L,L group (*n*=16)
Total cycle length (d)	26.0 (2.6)	25.8 (4.7)	28.4 (3.5)
Follicular phase length (d)	12.0 (1.9)	11.1[a] (4.7)	15.0 (3.2)
Luteal phase length (d)	13.0 (1.4)	13.7 (1.4)	12.4 (1.5)

H,H= High, High group
H,L= High, Low group
L,L= Low, Low group (controls)
[a] $P < 0.05$, H,L<H,H=L,L

for each subject.These data were examined using ANOVA and chi-squared tests respectively. The statistical analyses were performed using Stata Statistical Software 9 (release 9; Stata Corporation, College Station, TX).

Results

Basal characteristics of the subjects are shown in Table 1. Table 2 shows mean hormone levels on cycle day 3. No differences were found in age, Body Mass Index (BMI), smoking and history of smoking in packyears, duration of infertility and age of menarche between H,H group and H,L group. The age at which the mothers of the subjects experienced menopause was slightly lower in the H,H group compared to the other groups ($P < 0.05$).

Cycle duration
In the H,L group the mean duration of the follicular phase was shorter (11.1 days, range 3-22, $P = 0.047$) compared to the H,H group (12.0 days, range 10-16) and the control group (15.0 days, range 10-22). The duration of the follicular phase was determined by the peak LH value and did not include the day of the LH peak.The luteal phase did not significantly differ in length. Table 3 shows mean and standard deviations for all phases per group.

Hormone levels (see Table 4 and Figure 1)
Follicle Stimulating Hormone
Compared to the controls FSH was higher in the H,H group in all phases of the cycle and in the early follicular and early and late luteal phase in the H,L group. In the early follicular phase (EFP) and the late luteal phase (LLP) FSH was not significantly different in the H,H group in comparison to the H,L group, but in all other phases FSH was higher in the H,H group. The mean peak FSH level during the LH surge was significantly higher ($P < 0.001$) in the H,H group, compared to the other groups.

Luteinizing Hormone
LH in the H,H group was higher in the early follicular phase compared to the controls. In the mid follicular phase and in the early and mid luteal phase LH was higher in the H,H group compared to the H,L group ($P < 0.05$). In the late follicular and late luteal phase there were no differences between the 3 groups. LH peak values (LH surge) in the 3 groups were not significantly different.

Estradiol
The LH peak day showed lower estradiol levels in the H,H group compared to the

Table 4. Hormone values per cycle phase and per group. All values are mean and SD

	group	Early Follicular Phase	Mid Follicular Phase	Late Follicular Phase	LH surge	Early Luteal Phase	Mid Luteal Phase	Late Luteal Phase
FSH (IU/l)	H, H	13.3 (5.5)[b]	16.0 (8.2)[b,d]	10.4 (5.5)[b,d]	22.7 (10.6)[b,d]	11.3 (5.5)[b,d]	6.6 (4.7)[b,c]	5.9 (3.6)[a]
	H, L	10.4 (6.7)[b]	7.4 (3.8)	5.6 (2.1)	12.2 (7.7)	6.3 (1.2)[b]	3.7 (1.2)	4.7 (1.8)[a]
	L, L	4.9 (0.7)	4.9 (1.0)	3.7 (0.8)	8.9 (2.2)	4.1 (0.9)	2.7 (1.0)	3.1 (1.2)
LH (IU/l)	H, H	5.9 (1.1)[a]	6.0 (1.9)[a,c]	8.0 (2.1)	50.2 (27.1)[a]	10.0 (3.3)[c]	5.8 (3.1)[a,c]	4.1 (2.1)
	H, L	4.5 (2.2)	3.9 (1.4)	6.2 (2.6)	40.1 (27.2)	6.4 (5.4)	3.0 (1.9)	3.0 (1.0)
	L, L	3.7 (1.6)	4.5 (1.7)	6.4 (2.4)	32.6 (13.4)	6.8 (3.1)	3.3 (2.2)	2.9 (1.3)
E_2 (pmol/l)	H, H	88 (60)	154 (73)	466 (130)[c]	476 (122)[b,c]	350 (152)	438 (189)	262 (79)
	H, L	110 (36)	207 (58)	652 (259)	912 (422)	447 (310)	464 (160)	270 (129)
	L, L	113 (35)	178 (43)	525 (142)	726 (249)	330 (100)	361 (127)	233 (103)
Prog (nmol/l)	H, H	2.6 (0.9)[b]	1.9 (0.9)[a]	1.8 (0.5)	3.6 (0.9)	16.0 (8.3)	33.9 (18.6)	15.3 (7.5)
	H, L	1.9 (1.1)	1.7 (0.8)	1.6 (0.6)	3.3 (1.1)	19.4 (5.2)	42.7 (13.8)	19.1 (16.9)
	L, L	1.6 (0.7)	1.1 (0.5)	1.4 (0.9)	4.1 (2.0)	19.8 (6.3)	31.1 (13.0)	11.1 (4.9)
Inhibin B (ng/l)	H, H	22.2 (17.3)[b,c]	62.7 (33.9)[b,c]	61.2 (24.4)[c]	44.4 (18.3)[b]	46.4 (26.0)	15.4 (9.8)	11.8 (12.6)[a]
	H, L	71.5 (36.6)	102.8 (41.9)	85.0 (28.1)	62.3 (26.0)[b]	44.5 (33.3)	9.2 (3.1)	12.7 (11.0)
	L, L	96.2 (36.9)	113.1 (38)	82.5 (30.5)	128 (89.0)	70.4 (46.3)	19.4 (15.5)	24.5 (16.5)
Inhibin A (ng/l)	H, H	3.8 (1.3)[b]	7.8 (3.0)	26.9 (7.8)c	46.4 (11.1)[d]	40.5 (19.4)[d]	46.6 (16.2)	17.0 (4.2)
	H, L	5.9 (2.7)	10.2 (4.6)	43.9 (21.0)[a]	79.9 (29.1)[a]	70.3 (26.8)	53.5 (17.6)	16.9 (13.2)
	L, L	6.6 (2.0)	10.6 (4.9)	28.9 (8.6)	59.7 (19.1)	55.5 (14.9)	47.7 (15.4)	17.4 (10.4)

[a] different vs L,L; $P < 0.05$
[b] different vs L,L; $P < 0.01$
[c] different vs H,L; $P < 0.05$
[d] different vs H,L; $P < 0.01$

H,H (High, High: elevated basal FSH in screening and in the study cycle) group, n=11
H,L (High, Low: elevated basal FSH in screening and < 10 IU/l in study cycle) group, n=11
L,L (Low, Low, control) group, n=16

Table 5. Hormone values in the luteal and early follicular phase when defined according to 2nd day 3 value. All values are mean and SD.

	group	Early Luteal Phase	Mid Luteal Phase	Late Luteal Phase	Early follicular phase cycle 2
FSH (IU/l)	H, H (*n*=7)	7.6 (3.9)[b]	4.6 (3.4)[a]	5.9 (2.8)[b]	14.2 (3.8)[b,d]
	H, L (*n*=15)	9.4 (5.0)[b]	5.4 (3.8)[a]	5.0 (2.8)	6.6 (1.6)[b]
	L, L (*n*=16)	4.1 (0.9)	2.7 (1.0)	3.1 (1.2)	4.9 (0.8)
LH (IU/l)	H, H	8.8 (6.4)	4.5 (2.8)	3.8 (1.2)	6.3 (2.1)[b,d]
	H, L	8.0 (4.0)	4.3 (3.1)	3.3 (1.9)	3.7 (1.7)
	L, L	6.8 (3.1)	3.3 (2.2)	2.9 (1.3)	3.4 (1.4)
E_2 (pmol/l)	H, H	434 (337)	415 (116)	270 (128)	116 (48)
	H, L	376 (170)	474 (200)	262 (87)	204 (149)
	L, L	330 (100)	361 (127)	233 (103)	120 (40)
Prog (nmol/l)	H, H	21.9 (6.0)	47.4 (8.0)[a]	18.8 (11.2)	2.7 (1.3)
	H, L	15.7 (6.7)	34.0 (18.0)	16.6 (14.4)	2.2 (1.3)
	L, L	19.8 (6.3)	31.1 (13.0)	11.1 (4.9)	1.9 (2.4)
Inhibin B (ng/l)	H, H	54.6 (36.9)	7.2 (3.6)[a]	4.4 (4.1)[b,c]	42.7 (25.0)[b]
	H, L	39.0 (22.1)[a]	15.6 (7.8)	17.8 (11.7)	71.0 (38.5)
	L, L	70.4 (46.3)	19.4 (15.5)	24.5 (16.5)	97.2 (48.7)
Inhibin A (ng/l)	H, H	67.4 (30.7)	56.8 (18.9)	18.7 (13.9)	6.3 (3.0)
	H, L	47.5 (22.0)	44.3 (12.6)	15.1 (4.2)	13.1 (10.4)
	L, L	55.5 (14.9)	47.7 (15.4)	17.4 (10.4)	6.9 (2.9)

[a] different vs L,L; $P < 0.05$
[b] different vs L,L; $P < 0.01$
[c] different vs H,L; $P < 0.05$
[d] different vs H,L; $P < 0.01$

H,H (High, High: elevated basal FSH in screening and in the 2nd study cycle) group, *n*=7
H,L (High, Low: elevated basal FSH in screening and < 10 IU/l in 2nd study cycle) group, *n*=15
L,L (Low, Low, control) group, *n*=16

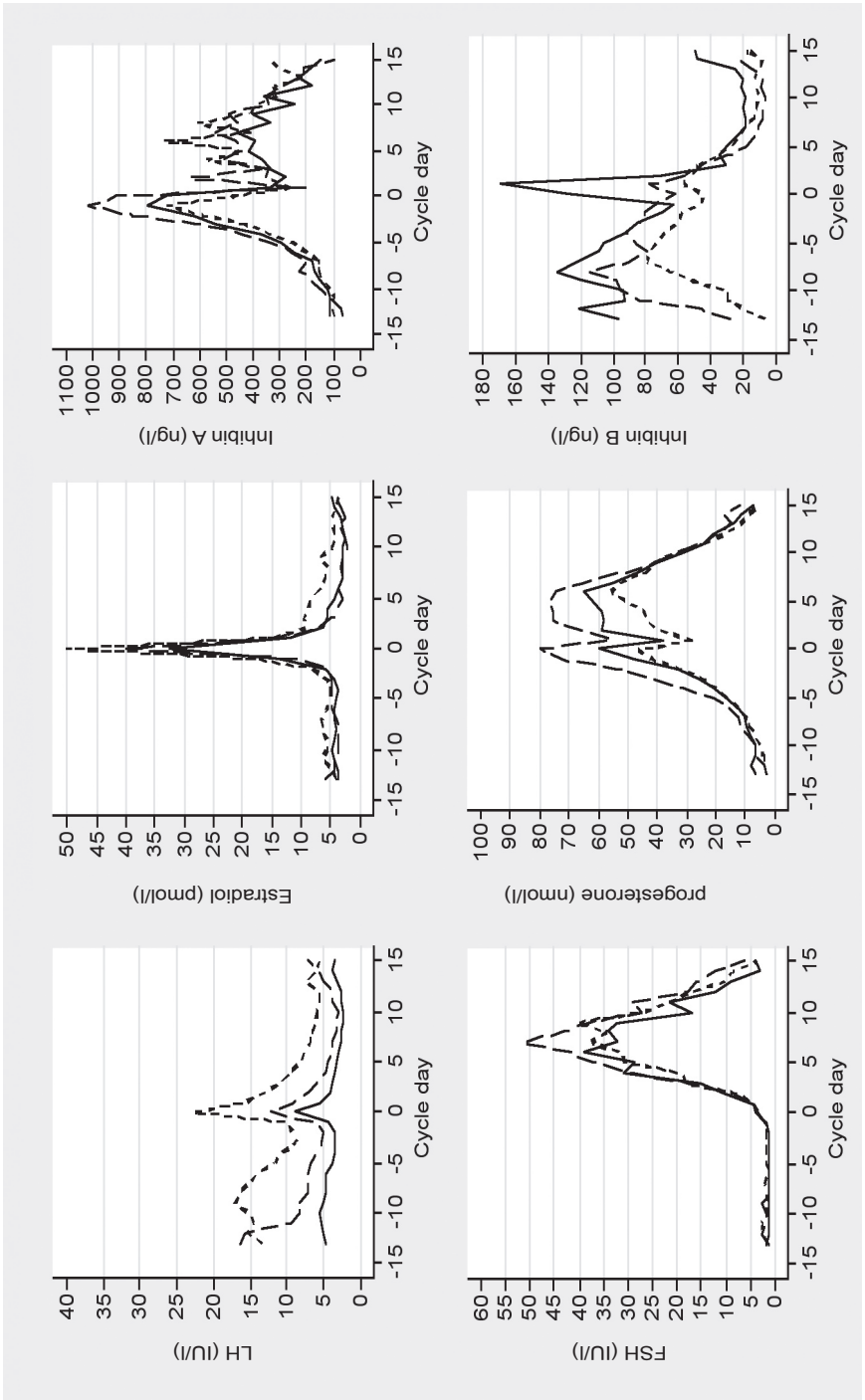

Figure 1. LH, FSH, estradiol, progesteron, inhibin A and inhibin B in one cycle of 11 patients with High basal FSH in screening and study cycle (H,H), 11 patients with High basal FSH in screening cycle but normal in study cycle (H,L), and 16 controls (L,L). The data are centered to the LH peak day. Controls are depicted by solid lines, H,L patients by broken lines, H,H patients by dense broken lines.

H,L group and controls. In the follicular and luteal phases we found no differences in E_2 between the 3 groups.

Inhibins

In the early follicular phase inhibin B was significantly lower in the H,H group compared to the other groups ($P < 0.01$ vs L,L group and $P < 0.05$ vs H,L group). Inhibin B was lower in the H,H group compared with the control group in the midfollicular phase ($P < 0.01$). At the time of the LH surge, H,H patiens and H,L patients show a decreased inhibin surge ($P < 0.01$).

Inhibin A was higher in the H,L group at the peri-ovulatory phase (late follicular phase, LH peak day and in the early luteal phase, $P < 0.05$). In the early follicular phase of the H,H group inhibin A was decreased ($P < 0.05$).

Progesterone

Progesterone was slightly higher in the early follicular and midfollicular phases in the H,H group, compared with the contols ($P < 0.05$). Progesterone did not differ in any other phase of the cycle.

Anti-Müllerian Hormone

Mean AMH in the control group was significantly higher compared to the H,H group and H,L group (Table 2). The correlation between day 3 AMH in the first and the second cycle was 0.84 in the H,H group, 0.82 in the H,L group and 0.87 in the control group.

Follicle growth and endometrial thickness

Growth velocity of follicles larger than 10 mm was 1.5 mm/day in the H,H group, 1.7 mm/day in the H,L group and 1.3 mm/day in the control group. This was not significantly different. Mean maximal diameter of the follicle at ovulation was significantly lower in the H,H group (18.8 mm) compared with the H,L group (21.6 mm)($P < 0.05$), but not lower than the control group (21.3 mm) ($P = 0.06$). Multifollicular growth was observed in all groups. However, in the H,H group 5/11 patients showed multifollicular growth (follicles > 10 mm). Only 1 patient in this group had two dominant (>16 mm) follicles, one ovulating 1 day after the other. In the H,L group 7/11 subjects showed multiple follicle growth. One patient in this group showed 3 dominant follicles of 19, 18 and 17 mm. respectively, of which 2 ovulated. In controls 5/11 had multiple follicle growth. Occurrence of growth of multiple dominant follicles was not statistically different between the groups. Endometrial thickness was not significantly different between the groups.

Intercycle variability

In order to evaluate the mechanisms causing intercycle variability of FSH levels in the early follicular phase, we also measured the hormone levels in the following cycle until day 5. We subsequently redevided the groups according to the day 3 FSH level of the second cycle and analysed the preceding luteal phase and early follicular phase of the second cycle. Table 5 shows the results. High FSH levels on day 3 were preceded by low levels of inhibin B in the preceding mid- and late luteal phase ($P < 0.05$). We found no significant differences in inhibin A in the preceding luteal phase when analysed in this way.

Discussion

Many studies described the endocrinology of ageing women and consistently found a rise in FSH levels in the follicular phase (Sherman *et al.*, 1976; Reyes *et al.*, 1977; Lee *et al.*, 1988; Klein *et al.*, 1996; Cameron *et al.*, 1988; Burger *et al.*, 1998). We report here the cyclical pattern of circulating concentrations of gonadotrophins, estradiol, progesterone and dimeric inhibins throughout the menstrual cycle in relatively younger women with elevated FSH levels compared to controls of the same age. Moreover, for the first time results of women with intermittently elevated FSH levels are described separately.

Women with repeatedly elevated early follicular phase FSH levels show many of the characteristic hormonal patterns seen in older women (Lee *et al.*, 1988; Klein *et al.*, 1996 and 2004; Danforth *et al.*, 1998; Welt *et al.*, 1999; Burger *et al.*, 1998). Serum FSH is elevated throughout all phases of the cycle together with lower inhibin B levels during the follicular phase, the LH surge and again in the luteal phase. Therefore also in these younger patients the elevated FSH is most likely the result of diminished ovarian feedback from a smaller available cohort of follicles in relation to limited oocyte reserve. This is supported by our finding that serum concentrations of AMH, secreted by small and intermediate antral follicles and considered to be a good marker for age related ovarian reserve (Visser *et al.*, 2006; Kevenaar *et al.*, 2006), were much lower than in controls. We also confirm the finding that inhibin levels in the early follicular phase are lower in these women which would further contribute to the notion that specifically limitations of ovarian peptide hormone feedback are involved in the regulation of pituitary gonadotropin secretion in relation to decreased ovarian reserve (de Koning *et al.*, 2000; Santoro *et al.*, 1999). Furthermore the inhibin B levels tended to be lower periovulatory and early in the luteal phase, which is also in line with earlier observations, probably reflecting a reduction in number and quality of the follicles growing during this part of the cycle (Muttukrishna *et al.*, 2000; Seifer *et al.*, 2002).

The inhibins selectively block pituitary GnRH-independent FSH secretion (Lambalk

et al., 1989; Rivier and Vale 1991). Therefore, the demonstrated lower inhibin levels are likely to be responsible for the monotropic rise of FSH. On the other hand we have shown that pituitary FSH and also LH responsiveness to GnRH is markedly increased in these women (de Koning *et al.*, 2000). This may explain our finding, that LH concentrations were also higher in almost all phases of the cycle, which also confirms similar findings by others (Lee *et al.*, 1988; Fitzgerald *et al.*, 1994; Ahmed Ebbiary *et al.*, 1994). The increased sensitivity of the pituitary to GnRH is possibly due to lower ovarian secretion of Gonadotropin Surge Inhibiting Factor (GnSIF, de Koning *et al.*, 2000). This biologically evident but sofar not biochemically characterized non-steroid hormone is secreted by granulosa cells of many species including the human, and reduces pituitary LH and FSH responsiveness to GnRH (de Koning 1995; Fowler *et al.*, 2003; Messinis, 2006). This hypothesis is supported by the recent observation of decreased GnSIF bioactivity in the spontaneous cycle in a group of women with poor response in IVF (Martinez *et al.*, 2002). Lower rates of GnSIF secretion could also explain the higher LH and FSH levels reached during the midcycle surge observed by us and others (Muttukrishna *et al.*, 2000) in women with raised basal FSH levels. Summarizing we suggest that elevation of gonadotropic hormones in women with elevated basal FSH is largely the net result of relaxation of ovarian inhibin restraint of GnRH-independent pituitary FSH, and relaxation of the restraint by GnSIF on GnRH-dependent FSH and LH secretion rather than changes in ovarian steroid hormones.

The slightly lower estrogen levels during the LH surge in the patients with the persistently elevated FSH levels are probably a reflection of either quantitatively and/or qualitatively diminished dominant follicle development (Seifer *et al.*, 1996, 2002; Welt *et al.*, 2005).

We found slightly elevated progesterone levels in the early follicular phase. So far this has been described in only one other study describing the follicular phase endocrine events in previous IVF low responders (Beckers *et al.*, 2002) which was interpreted as the result of remaining secretion from the corpus luteum of the previous cycle. Remarkably luteal phase progesterone secretion prior to elevated cycle day 3 FSH was also higher than in the controls, so possibly prolonged weaning from higher earlier elevated levels may have continued into the next follicular phase. In the past we have suggested that in women showing perimenopausal limited ovarian reserve shortening of the follicular phase length results from some merging of the follicular phase into the luteal phase of the previous cycle (Lambalk *et al.*, 1998b), in particular in view of the fact that growth speed and end size of the developing dominant follicle as shown in this study and previously by others is not different (Klein *et al.*, 2002; van Zonneveld *et al.*, 2003). However, in the current study we found no shorter length of the follicular phase when basal FSH was elevated. Similarly in the study by Beckers follicular phase cycle length was not shortened (Beckers *et al.*, 2002). In our

view a more likely explanation for the elevated early follicular phase progesterone levels would be some premature luteinization due to the higher LH levels. This is supported by the fact that both progesterone and LH levels normalized in women with a temporary normalization of early follicular phase FSH levels.

Our study provides novel detailed information with regard to the reproductive endocrine profile in women with temporarily normalized basal FSH levels. In our study all these patients showed low AMH levels comparable to levels found in women with persistingly elevated FSH. This indicates that variably elevated basal FSH levels are also a reflection of limited ovarian reserve. Therefore, the fact that the FSH levels were still slightly higher compared to controls was not surprising. On the other hand, with regard to secretion of the other hormones, the daily measurements revealed remarkable differences. The concentrations of LH, estradiol and progesterone became comparable to controls. Literature often but not invariably reports early follicular phase elevations of estradiol as a reflection of active ovarian feedback from protracted development of a dominant follicle. This explains the relatively normal FSH levels in patients with limited ovarian reserve and the somewhat shorter follicular phase (Evers *et al.*, 1998). We found indeed a tendency of estradiol to be higher and a shorter follicular phase in these patients compared to controls and women with consistently elevated basal FSH. Therefore it could be that the temporally normalization of FSH is a reflection of advanced follicular development. According to our observations the normalization of FSH was typically accompanied by normalization of the secretion of inhibin B, not only during the early follicular phase but already during the mid and late luteal phase of the preceding cycle. Inhibin B, the product of developing non-dominant antral follicles, can be considered as a good marker of the number of follicles that can be recruited by FSH, and as such it may be a reflection of the size of the cohort available in that particular cycle (Lockwood 2004; Kwee *et al.*, 2003). This implies, that according to our findings, the variability of follicular development not only with respect to timing but also in terms of quantity may be an important cause of the variability of early follicular phase values.

A noteworthy finding in the patients with temporarily normalized basal FSH was the observation that inhibin A levels were increased during the late follicular, the periovulatory and the early luteal phase. Other publications reported higher inhibin A levels pre-ovulatory in older cycling women (Klein *et al.*, 2004) and in the mid follicular phase (Reame *et al.*, 1998). This could be a result of increased quantity and/or a higher stimulation of the granulosa cels. We suggest that an increase in number of inhibin A producing cells, possibly because of more than one developing follicle is a good explanation for the higher inhibin A levels. Indeed 7 out of the 11 H,L patients showed development of more then one growing follicle (> 10 mm). So probably the temporarily larger cohort of FSH recruitable follicles, evidenced

by the normal inhibin B levels in the beginning of the cycle, in combination with the slightly higher FSH levels, creates circumstances that favour selection and development of multiple dominant follicles. Relevant in this respect is that higher basal FSH levels were found in older women in whom multiple follicle development occurred (Beemsterboer *et al.*, 2006).

The question is if this notion may be of some benefit for the clinical approach towards these patients in particular with IVF. Based on our finding, that suggests presence of a temporary larger cohort when basal FSH normalizes, one would indeed expect a better ovarian response upon hormonal hyperstimulation as suggested earlier (Lass *et al.*, 2000). The only study published in this respect (Abdalla and Thum 2006) compared IVF oocyte yield and pregnancy result in 39 patients who were stimulated in one cycle with elevated FSH and another cycle with normal FSH, and found no differences. In this retrospective study however, samples used for the estimation of the FSH were often not collected in the beginning of the same IVF stimulation cycle from which outcome was measured. Our study indicates that in women with low ovarian reserve the intercycle variability of FSH is high, which means that if a basal FSH value is to be used to determine the acute size of the cohort, it should be a value in the early follicular phase of the stimulation cycle. A prospective trial with uniform prevention of premature luteinization prevention (short protocol GnRH agonist or a GnRH antagonist) and standard gonadotropin stimulation is needed to establish or rule out the possible usefulness of such a strategy.

In conclusion, this study shows that relatively younger women with either intermittently or constantly elevated day 3 FSH levels have a diminished ovarian reserve shown by persistently low AMH levels. The endocrine profile in patients with consistently elevated basal FSH resembles that in published data from older women. However, patients who present with elevated early follicular phase FSH but normal FSH in the subsequent cycle are characterized by normalization of inhibin B in the preceding luteal phase, suggesting a temporary increase of the available cohort.

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3 Elevated FSH concentrations in imminent ovarian failure are associated with higher FSH and LH pulse amplitude and response to GnRH

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Abstract

Background: Imminent Ovarian Failure (IOF) in women is characterized by regular menstrual cycles and elevated early follicular phase Follicle Stimulating Hormone (FSH).

Material and Methods: This study explored underlying neuroendocrine causes of elevated FSH on day 3 of the menstrual cycle. The characteristics of episodic secretion of FSH and LH, the pituitary response to gonadotrophin-releasing hormone (GnRH), plasma oestradiol, and dimeric inhibin A and inhibin B on day 3 were compared in 13 women with elevated FSH concentrations (> 10 IU/l) and 16 controls.

Results: FSH amplitudes were higher in the IOF group than in the controls ($P<0.0001$). The FSH pulse frequency did not differ between groups. The FSH response to GnRH was higher in the IOF patients than in the controls ($P<0.0001$). Mean LH, LH amplitude and LH response to GnRH were higher in the IOF group, but LH pulse frequency did not differ between the groups. Concentrations of inhibin A and inhibin B were lower in the IOF group, while oestradiol showed no differences.

Conclusions: We concluded that in women with IOF, the pituitary is more sensitive to GnRH. This leads to higher FSH and LH pulse amplitudes which underlie elevated FSH concentrations in the early follicular phase.

Introduction

Early follicular phase FSH concentrations are often used to predict the outcome of IVF (Muasher *et al.*, 1988; Scott *et al.*, 1989; Licciardi *et al.*,1995; Scott and Hofmann, 1995). Elevated day 3 FSH concentrations are well correlated with a poor response in ovarian stimulation and low pregnancy rates (Scott and Hofmann, 1995). These patients are often referred to as having imminent or incipient ovarian failure (IOF) (Jones *et al.*, 1986; Cameron *et al.*, 1988; Buckler *et al.*,1991).

It is generally assumed that early follicular phase FSH, at the time of recruitment of follicles, is elevated due to diminished ovarian feedback of steroids and inhibins (Sherman and Korenman 1975; Lee *et al.*, 1988; MacNaughton *et al.*, 1992). Inhibins are dimeric proteins that selectively inhibit FSH secretion. Inhibin A is composed of a common α subunit and a ßA subunit, and inhibin B consists of an α subunit combined with a ßB subunit. In recent years studies on the differential secretion of inhibin A and inhibin B in the menstrual cycle (Groome *et al.*, 1996) and in-situ hybridization studies (Roberts *et al.*, 1993) have shown that inhibin A appears to be primarily secreted by the mature follicle and corpus luteum. Inhibin B appears to be secreted by smaller preovulatory follicles. Decreased concentrations of both inhibin A and inhibin B can theoretically contribute to high FSH concentrations in the early follicular phase.

It is not fully clear, however, what compounds, attributing to the rate of pituitary FSH secretion, are altered in patients with IOF. Hypothalamic causes for elevated FSH concentrations could induce an altered release mode (more pulses) of pulsatile GnRH as in mothers of dizygotic twins (Lambalk *et al.*, 1998). At the level of the pituitary a higher sensitivity to the GnRH pulses might explain the elevation of FSH. FSH and LH are released in a pulsatile manner. Studying episodic gonadotrophin release enables observation of dynamic changes in the FSH release of patients with IOF. Since pulsatile LH is considered to be a good representation of the episodic activity of the hypothalamic GnRH pulse generator, a detailed analysis of its episodic gonadotrophin secretion in combination with information on the LH and FSH response to a GnRH challenge, will potentially reveal responsible mechanisms.

The aim of this study was to evaluate the hypothalamic and pituitary contribution to the elevated FSH concentrations in women with IOF. Therefore, the pulsatile release of FSH and LH on day 3 of the menstrual cycle was examined and the subsequent pituitary response to GnRH in patients with IOF and controls was evaluated. The role of the ovary as a potential cause of the differences in gonadotrophin secretion was also evaluated by concomitant measurement of the concentrations of oestradiol, inhibin A and inhibin B.

Materials and methods

Subjects

Patients, referred to our infertility clinic, were all screened on day 3 of the menstrual cycle for possible elevated FSH for a period of 3 years between January 1995 and January 1998. All patients with a FSH value of ≥10 IU/l, were asked to participate in the study. In our IVF clinic patients with basal FSH values > 10 IU/l show very poor outcome with respect to numbers of oocytes retrieved and pregnancy rate.

Controls were either patients referred to our clinic for reversal of tubal ligation or volunteers recruited by advertisement, with cycle day 3 FSH values <10 IU/l. There was no attempt to match controls and patients for age, weight or other demographic characteristics. Patients in both the study population and the control group had regular menstrual cycles of 21-35 days, no hot flushes and no medical or hormonal treatment during at least 3 months prior to the study. Controls had no history of infertility. The local ethics committee approved the study. All subjects gave informed consent.

Study design

Serial blood samples were collected on day 3 of a later menstrual cycle (study cycle). An indwelling catheter was placed in a forearm vein and for 6 h. blood was drawn into heparinized tubes every 10 min. Sampling started between 08:00 and 09:00. Immediately after the last sample a GnRH challenge with an i.v. injection of 100 µg GnRH (HRF; Wyeth, Hoofddorp, The Netherlands) was given and three additional blood samples were taken after 30, 60 and 90 min. The basal body temperature (BBT) of all subjects was measured during the study cycle.

Hormone measurements

LH and FSH were measured in duplicate by commercially available immunometric assays (Amerlite; Amersham, Bucks, UK). The lower limit of detection was 0.3 IU/l for LH and 0.5 IU/l for FSH. The assays were calibrated against the 1st International Reference Preparation (IRP) 68/40 and 2nd IRP 78/549 for LH and FSH respectively. Of each individual, all samples were analysed in the same run for each hormone. The inter- and intra-assay coefficients of variation (CV) were < 9% and 5% for LH and FSH.

Inhibin A and Inhibin B were measured in duplicate by ultra-sensitive two-site enzyme immunoassays (Serotec, Oxford, UK). The development of these commercially available assays was based on the work of Groome *et al.* (Groome *et al.*, 1994, 1996). The lower limit of detection was 3 pg/ml for inhibin A and 15 pg/ml for inhibin B. The inter-assay CV was < 9% for both inhibin A and inhibin B. Oestradiol was measured by radioimmunoassay (Sorin Biomedical, Sallugia, Italy) with a lower limit of detection of 18 pmol/l and an inter-assay CV of < 11%. For

data analysis, values below the lower limits of detection were assigned the value of assay sensitivity.

Pulse analysis

Pulse analysis was carried out with a computerised version of a previously developed and validated method (Lambalk *et al.*, 1985; Scheele *et al.*1987). The algorithm is valid for replicate repeated measurements of LH and FSH with a chance of $< 5\%$ to indicate non-existing pulses as a pulse in series of 100 samples taken from pooled serum. This method is particularly of value in detecting episodic secretion of hormones with relatively long half-lives because pulses are indicated when a significant rise occurs without the requirement of a subsequent decline. Nadirs preceding the pulses are indicated as marker points in the hormone patterns rather than the pulses themselves.

Statistical analysis

The patients had to have elevated day 3 FSH in the screening as well as in the study cycle. The controls had normal FSH concentrations in both cycles. For each subject, the mean concentrations of LH and FSH, the mean pulse amplitude over the 6 h period and the frequency of LH and FSH pulses per 6 h were calculated. The maximal gonadotrophin increment was taken as a parameter for the response to the GnRH challenge. The non-parametric Mann-Whitney *U*- test was used for differences between groups. $P < 0.05$ was considered to be statistical significant. Spearman rank correlations were calculated for overall relation between mean FSH concentrations and inhibin A and inhibin B.

Results

Baseline characteristics

There were 13 patients in the IOF group, 16 in the control group. Age, body mass index (BMI), exposure to smoking in pack years (number of years of daily intake of 20 cigarettes), and current smoking were similar for the groups (Table 1). All controls were ovulatory as evidenced by biphasic BBT charts. One patient in the IOF group had a monophasic BBT. Menstrual cycle length and follicular phase length were significantly shorter in the IOF group compared to controls.

Gonadotrophins

Figure 1 shows examples of the secretory gonadotrophin patterns and responses to GnRH from each group.

Table 1. Baseline characteristics of patients suffering from imminent ovarian failure (IOF). Values are given as mean ± SD.

	IOF group *n*=13	Control group *n*=16
BMI (kg/m^2)	21.4 ± 1.6	24.2 ± 4.5
Age (years)	35.9 ± 4.2	34.4 ± 3.7
Cycle length (days)	26 ± 2.6*	28.4 ± 3.5
Follicular phase (days)	11.5 ± 1.9**	14.6 ± 3.2
Packyears[a]	8.5 ± 7.7	4.1 ± 6.4
Currently smoking (*n*)	6	4

[a]Pack years = no. of years of daily intake of 20 cigarettes.
*Cycle length in IOF group was shorter than in the controls (P<0.05).
**Follicular phase length in IOF group was significantly shorter than in the control group (P<0.005).

FSH

The characteristics of pulsatile FSH secretion are summarized in Figure 2. The mean FSH was significantly higher in the IOF group (15.4 ± 5.3 IU/l), than in the control group (4.4 ± 1.1 IU/l, P <0.0001). The FSH pulse amplitude was higher (P < 0.0001) in the IOF group (1.20 ± 0.56 IU/l) compared with the controls (0.37 ± 0.11 IU/l). The IOF group showed a highly significant (P <0.0001) increase in FSH response to GnRH (10.3 ± 5.5 IU/l) compared to the control group (2.3 ± 1.3 IU/l). The pulse frequencies did not differ.

LH

Figure 3 summarizes the results for pulsatile LH secretion. Mean LH in the IOF group (5.7 ± 2.0 IU/l) was significantly higher (P <0.0001) than in the control group (2.5 ± 0.9 IU/l). The LH pulse amplitudes in the IOF group (1.77 ± 0.71 IU/l) were significant higher (P <0.001) than in the control group (0.92 ± 0.39 IU/l). There were no differences in the LH pulse frequency. As with FSH, the maximal LH increment to GnRH was higher (P <0.001) in the IOF group (19.9 ± 9.1 IU/l) than in the controls (9.5 ± 3.4 IU/l).

Oestradiol and inhibins

Figure 4 shows the results of oestradiol, inhibin A and inhibin B measurements. Patients in the IOF group showed oestradiol concentrations similar to the control group. In the IOF group, serum concentrations of inhibin A and inhibin B were significantly lower than in the control group. Mean inhibin A in the IOF group was 5.5 ±6.1 pg/ml and in the control group 7.9 ± 3.2 pg/ml, P <0.01. Mean inhibin B

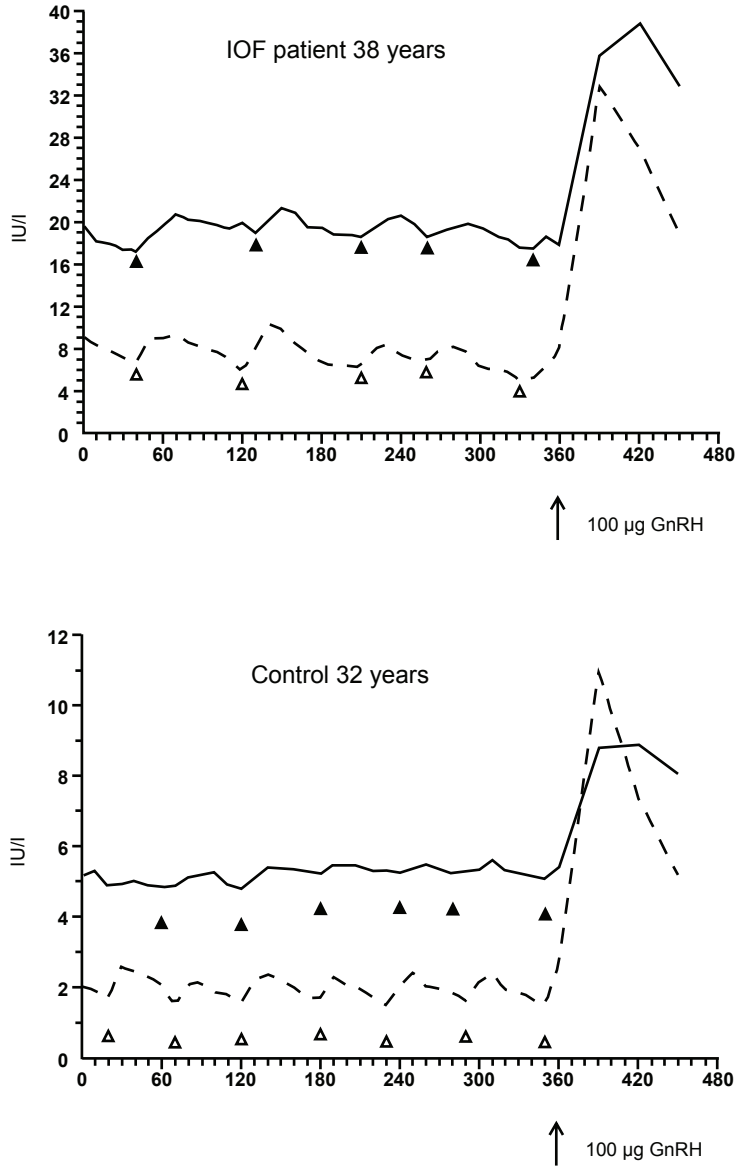

Figure 1. Examples of LH (dashed lines) and FSH (solid lines) secretion patterns and responses to gonadotrophin-releasing hormone (GnRH) in a woman with imminent ovarian failure (IOF) (upper panel) and a control patient (lower panel) on cycle day 3.
▲ = start of an FSH pulse, ▵ = start of an LH pulse

in the IOF group was 52.9 ± 44 pg/ml, and in the control group 81.8 ± 40 pg/ml, $P < 0.05$. There was an inverse correlation between inhibin A and FSH (r = -0.71, $P < 0.001$). An inverse correlation was also found (r = -0.63, $P < 0.001$) for inhibin B and FSH.

Discussion

Elevated FSH in women with IOF can be explained by the presence of higher FSH pulses. These larger FSH pulses result from an increase in pituitary response to GnRH. Moreover, there appears to be a hitherto unreported, subtle concomitant rise of LH on day 3 of the cycle. The secretory dynamics of LH appear to be a copy of FSH with similar higher pulse amplitudes and responses to GnRH.
These findings contrast with a previous study of the episodic FSH secretion in mothers of hereditary dizygotic twins. In those we found elevated FSH concentrations in association with an increased number of FSH pulses, without changes in the response to GnRH and without alterations in LH (Lambalk *et al.*, 1998). This indicates that in twin mothers the origin of elevated early follicular phase FSH is pituitary or supra pituitary, whereas elevated FSH in imminent ovarian failure is of ovarian origin.

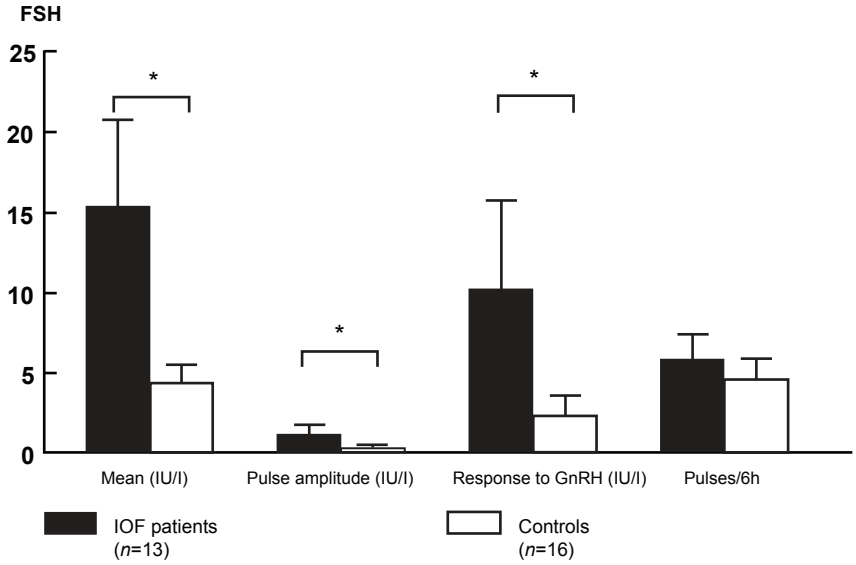

Figure 2. Means and SD of different parameters of episodic FSH secretion on cycle day 3 In imminent ovarian failure (IOF) patients compared with controls. *$P < 0.0001$

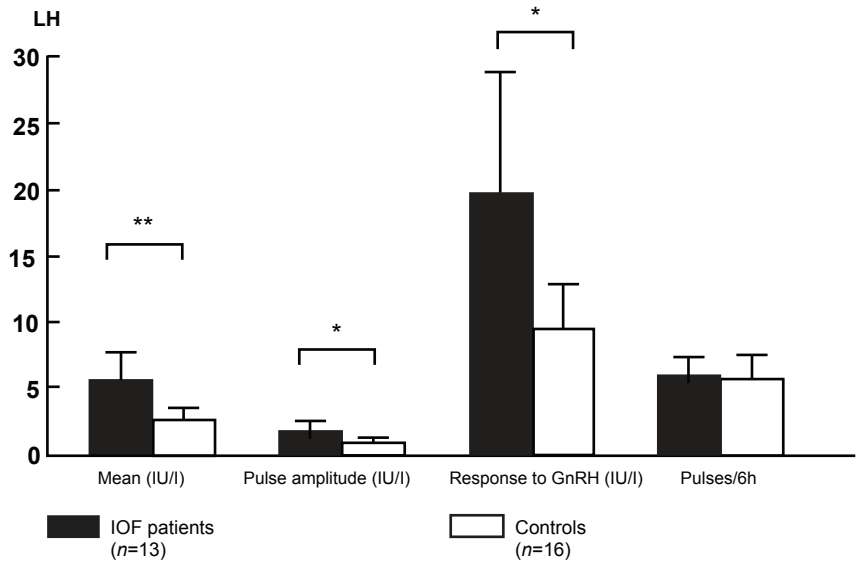

Figure 3. Means and SD of different parameters of episodic LH secretion on cycle day 3 in imminent ovarian failure (IOF) patients compared with controls. *$P < 0.001$; **$P < 0.0001$

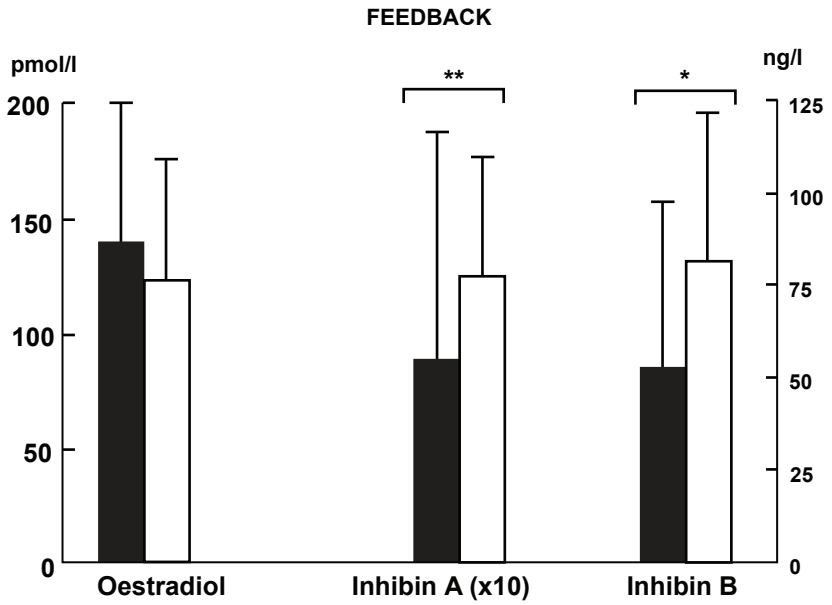

Figure 4. Means and SD of oestradiol, inhibin A and inhibin B on cycle day 3 in imminent ovarian failure (IOF) patients compared with controls. *$P < 0.05$; **$P < 0.01$

These contrasting findings, in seemingly identical conditions, i.e. elevated early follicular phase FSH, underscore the importance of investigating the dynamics of the episodically secreted gonadotrophins.

Only a few others have studied the dynamics of gonadotrophin secretion in relation to the luteal follicular transition (Hall *et al.*, 1992) and in reproductive ageing (Wilshire *et al.*, 1995; Klein *et al.*, 1996b; Reame *et al.*, 1996). The increase in LH pulse frequency in this stage of the menstrual cycle, due to increased GnRH pulsatility, shows the importance of GnRH action in the increase of FSH in the early follicular phase (Hall *et al.*, 1992).

The age-related increase of FSH concentrations, is associated with enhancement of pulsatile LH secretion, particularly in the LH pulse amplitudes as reported by Reame (Reame *et al.*, 1996). This is in full agreement with our observations and we can add that greater pituitary responsiveness to GnRH is most probably responsible for this. Klein *et al.* (Klein *et al.*, 1996) in a smaller study, were not able to detect differences in the endocrinology of the early follicular phase in older and younger cycling women in their pulsatility study. Moreover, Wilshire *et al.* (Wilshire *et al.*, 1995) reported no differences in LH pulse amplitudes and number of pulses during the early follicular phase in younger versus older women. In a frequent sampling study of LH in the late follicular phase Matt (Matt *et al.*, 1998) found a lower number of LH pulses in older women, which may indicate a slowing of the GnRH pulse generator in that phase. This is in line with our earlier observations in younger versus older postmenopausal women (Lambalk *et al.*, 1997). Whether a slowing of the GnRH pulse generator demonstrated in the late follicular phase is responsible for a paradoxical increased level of FSH in the early follicular phase is highly questionable (Lambalk *et al.*, 1989).

The GnRH challenge tests have been used by a few other authors to study gonadotrophin secretion dynamics in reproductive ageing. Fujimoto (Fujimoto *et al.*, 1996) in contrast to our findings, described a lower gonadotrophin response to GnRH in older women. This apparent discrepancy may be explained by the fact that Fujimoto used age as a primary variable, while we compared the results of IOF patients with a control group. Muasher *et al.* found that the FSH/LH response to GnRH correlates with results in IVF (Muasher *et al.*, 1988). In this study, identical to our results, higher gonadotrophin responses in patients with elevated FSH were observed. Finally, an increased response of both LH and FSH to GnRH was described in a study performed by Schmidt *et al.* (Schmidt *et al.*, 1996) in perimenopausal women with irregular cycles between 10-90 days, but no differences in response to GnRH were observed in older subjects with a regular cycle.

Ovarian feedback seems to play a role in alterations of early follicular phase FSH secretion. There were no differences between the groups in oestradiol concentrations in our study. This is in line with observations by others (Sherman and Korenman,

1975; Lee *et al.*, 1988; Buckler *et al.* 1991). In the current study, the elevated cycle day 3 FSH concentrations were found to be associated with lower concentrations of inhibin A and inhibin B. A number of studies have indicated an inverse relation between inhibin B and calender age (Klein *et al.*, 1996a) and a relationship between low inhibin B and poor outcome in assisted reproduction (Seifer *et al.*, 1997). One study (Reame *et al.*, 1998), also showed older cycling women to have lower follicular phase inhibin B. Recently, inhibin B was reported to be lower in the early follicular phase of older women (Welt *et al.*, 1999), together with lower inhibin A on the day after the LH peak. In that study FSH concentrations were slightly higher in the early follicular phase of older (>35 years) women. It is believed that lower inhibin B concentrations signify a decline in size of the available cohort of follicles, and increased early follicular phase FSH concentrations probably represent the same. By focusing on differentiation between high and normal FSH concentrations our results clearly indicate towards a role of deficient inhibin A. Inhibin A is predominantly secreted in the luteal phase (Groome *et al.*, 1996). Therefore, early follicular phase rise of FSH may at least in part result from some luteal phase deficiency of the previous cycle as put forward by Danforth *et al.* (Danforth *et al.*, 1998). They observed a good inverse relation between luteal inhibin A concentrations and day 3 FSH values. In addition it has been shown by Seifer *et al.* that cultured luteinized granulosa cells of women with high FSH concentrations produce less inhibin A (Seifer *et al.*, 1996).

Activins were not measured in this study. Inconsistent data are available on the role of activins in pituitary stimulation. Some authors (Ying *et al.*,1988) report that activins are capable of direct pituitary FSH stimulation, whereas others (Katayama and Conn, 1994) question this. Nevertheless, slightly higher activin A concentrations are found in older premenopausal women (Reame *et al.*, 1998). These data were confirmed by another study (Santoro *et al.*,1999). So far, a role of activin A as a classical hormone involved in gonadal function remains unclear (Harada *et al.*,1996).

Based on the current understanding of inhibin physiology (Hayes *et al.*, 1998), it is not possible to explain fully the exaggerated GnRH-induced gonadotrophin response in patients with IOF. Inhibins selectively inhibit FSH secretion, so the decreased inhibin A and inhibin B may account for the increased FSH response, but not for the higher LH response. The concentrations of oestradiol and progesterone (data not shown) were not lower in IOF patients. Therefore, a decline in the activity of other ovarian regulators might be involved in the loss of negative feedback across the cycle. We speculate that a possible mediator of reproductive ageing is loss of gonadotrophin surge inhibiting factor (GnSIF) activity. This ovarian peptide keeps the pituitary in a low state of responsiveness to GnRH (de Koning, 1995; Fowler *et al.*, 1995; Balen 1996). Changes in GnSIF activity are thought to play a role in the generation of the midcycle LH surge. So far, there is no reliable assay available to measure GnSIF serum concentrations. GnSIF bioactivity is present in human

follicles (Fowler *et al.*, 1995) and concentrations vary across the follicular phase (Fowler and Templeton, 1996).
In conclusion, elevated day 3 FSH concentrations in women with Imminent Ovarian Failure result from a pituitary more sensitive to GnRH, leading to higher FSH and LH pulse amplitudes and an increased response to GnRH. Decreased feedback by ovarian inhibin may play a central role.

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4 Assessment of ovarian reserve. Ovarian biopsy is not a valid method for the prediction of ovarian reserve

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Abstract

Background: The evaluation of ovarian reserve, often critical for the elderly infertile woman, is notoriously difficult and inaccurate. The place of ovarian biopsy in this evaluation has been hotly disputed for three decades, but not resolved. To examine the feasibility of ovarian biopsy for this purpose, a project was designed to estimate the total number of oocytes in a human ovary and investigate whether any biopsy regimen is representative of the follicular reserve in an individual.

Material and Methods: Ovaries removed from patients of reproductive age during operations not involving ovarian pathology were utilized to count the number and type of follicles found in multiple biopsies of 2 and 5 mm and in the whole ovary.

Results: Representative results taking into account the total number of follicles found in the whole ovary showed that predicted values based on the biopsies were extremely varied.

Conclusion: We concluded that due to the huge variation in the distribution of follicles across the surface of the ovary, there is no place for this procedure in clinical evaluation of reproductive ageing in the individual patient.

Introduction

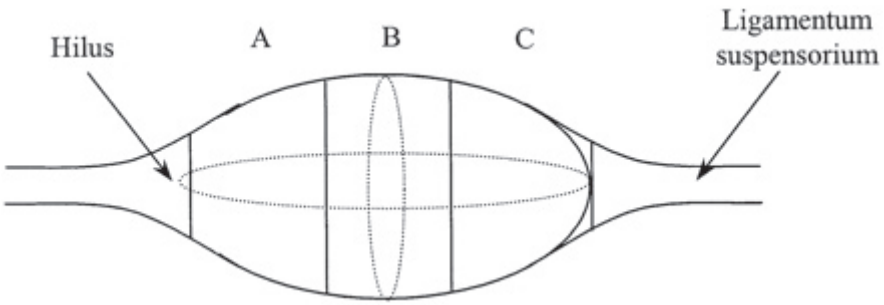
This contribution to the debate on the use of ovarian biopsies for assessing ovarian reserve (Lass, 2004; Sharara and Scott, 2004) is based on our experience while examining whether laparoscopic ovarian biopsy for such a purpose was justified.
It is obvious from the literature that it is very difficult to predict ovarian reserve and the age of menopause with accuracy. An accurate prediction would be invaluable for several reasons: planning pregnancies for those at risk of premature ovarian failure (POF) and those likely to have a later menopause, and for the determination of the status of the ovary for preservation before cancer treatment.
As life expectancy has increased, so has the desirable age for conception. This has led to the need for a way of forecasting the age when the follicular store will be extinguished. Since there is a continual fall in follicle number, with no method of replacing lost follicles, estimation of follicle number and rate of decline seems the most logical method of forecast. This is not as easy as it sounds as obtaining a measure of the total number of oocytes in the ovaries is not possible without removing them. In addition, the rate of follicle loss is not constant (Faddy *et al.*, 1992; Gougeon, 1996).
Many techniques and observations have been used to attempt to estimate ovarian reserve and predict those with a poor chance of success in ART: age, FSH, inhibin, anti-Müllerian hormone and estradiol concentrations; dynamic tests using GnRH agonist, FSH or clomiphene citrate; measurement of ovarian volume, ovarian antral follicle count; and, the subject of our investigation, ovarian biopsy.
We designed a project to collect ovaries during surgery from women in the fertile period of life, taking systematic biopsies and counting the number of follicles in the biopsies and in the whole ovary in order to examine if one or more biopsies could be used to predict the grand total reliably.

Material and Methods

Patients
Ovaries removed from five patients of varying ages for reasons not involving ovarian pathology were utilized in this study. All were originally cycling women whose ages ranged from 30 to 46 years. The reasons for oophorectomy were unexplained abdominal pain, breast cancer and female to male transsexuality.

Procedure
After surgery, the surface of the ovary was removed and divided into 12 segments and, from each segment, a biopsy of 2 mm diameter was taken with a Schumacher or other instrument. The region closest to the hilus was labelled A, the middle as B and

the outermost region (closest to the ligamentum suspensorium) as C (see Figure). From each part, four biopsies were taken at regular intervals and named A1, A2, A3, A4, B1, etc. At the most distal region from the hilus, a 5 mm biopsy D was taken with Palmer forceps. All biopsies and the ovary were measured. The ovaries were fixed and embedded in paraffin wax.



Histology

All biopsies were sliced at 5 µm intervals, mounted on slides and stained with haematoxylin and eosin.

All sections were examined under the light microscope at 100x magnification, and the number of primordial, early primary, 1–2 layers of granulosa, 3–4 layers, multilayered and Graafian (antral) follicles counted (Faddy *et al.*, 1987). Only follicles where the nucleus could be seen were counted in order to prevent counting any one follicle twice. An attempt was made at following follicles from section to section to reduce the likelihood of double counting or of missing follicles with indistinct nuclei.

The remaining ovary was sliced at 5 µm intervals. Sections were sampled at regular intervals of one in 25 and stained. Every 50th section was examined, counted and grouped using the same method as for the biopsies, but all visible follicles were counted irrespective of the presence of nuclei. The distance between sections (250 µm) ensured that no follicle was counted twice. This was the reference for the total number of follicles in the ovary.

Analysis

Whole ovary. The maximum size of a primordial follicle is 50 µm. As sections were taken at 5 µm but only counted every 50th, potentially there were small follicles in the 250 µm space between observed sections. Each primordial follicle appears in up to 10 subsequent sections. This means only one primordial follicle was observed for every five that may have been present. The following conversion was therefore used

to interpret the data (Zuckerman and Weir, 1977).
In the case of primordial follicles, with a maximum diameter of 50 μm, the correction was as follows:

5 μm/50 μm x 50 x no. of follicles/section

In addition, the number of follicles within the biopsies must be added to the total.

Biopsies. The quantity of follicles recorded in the biopsies is an absolute number and no correction factor was applied. In order to make predictions regarding the number of follicles within the ovary, the surface area of the biopsy was taken as a proportion of the surface area of the ovary. This is because the cortex of the ovary lies at the surface and the medulla is virtually devoid of follicles.
The area of the biopsy was calculated as a circle (πr^2) and the surface area of the whole ovary was calculated as an ellipsoid as illustrated below. The area of the hilus was subtracted from the total area of the ovary, as it is known to be devoid of oocytes. The area of the hilus was assumed to equal to 5 mm^2 in all cases.

$$\text{The surface area of an ellipse} = 4\pi\left(\frac{r_1^2 r_2^2 + r_1^2 r_3^2 + r_2^2 r_3^2}{3}\right)^{1/2}$$

Where r_{1-3} = the radii in the three axes of the ellipse.

The surface areas of the 2 and 5 mm biopsies were 3.14 and 19.64 mm^2, respectively.

Results

Although five ovaries were collected, following a thorough examination of three of them it became very apparent that even numerous biopsies could not predict the total number of follicles in an ovary. We therefore abandoned the detailed examination of further ovaries as this was thought to be a waste of considerable time and effort without a worthwhile return.
We present here the results from three ovaries. The first patient who donated an ovary (ovary A) to the study was 33 years old with two children and had a regular menstrual cycle at the time of oophorectomy. The reason for surgery was intractable, very severe lower abdominal pain, unresponsive to any treatment given. After extensive, multidisciplinary discussions, it was decided to meet the patient's request to perform a hysterectomy and bilateral oophorectomy. All organs removed were found to be perfectly normal on pathological examination.
The other ovaries (ovaries B and C) were from women aged 33 and 34 years,

respectively, who underwent oophorectomy because of female to male transsexuality. Both had received testosterone (250 mg i.m. once every 2 weeks) treatment for 3 months prior to the surgery. Their ovaries were enlarged and had a polycystic appearance.

Table I summarizes the numbers of follicles found in each of the biopsies and those found in the whole ovaries. The total counts in the whole of ovaries A and C entirely matched with their age, fitting exactly on the curve of normal values constructed by Faddy *et al.* (1992). Ovary B, however, was deviant, and the number of follicles counted was equivalent to that found in women around 40 years of age. The numbers of follicles varied greatly across the various biopsies. A summary of predictions of follicle numbers based on biopsies is also given in Table I.

There was again great variation in the predicted total number, based on transformation of the biopsy count to the whole ovary. The average predicted number found in the 12 biopsies of 2 mm was also very deviant from the whole counts. Taking into account the 'true' total number of follicles found in the whole ovary, the predicted values based on the biopsies varied tremendously from +223% to –97% in ovary A, +3619% to –100% in ovary B and +492% to –42% in ovary C.

Discussion

Steele *et al.* (1970), the first to advocate the use of ovarian biopsy for the investigation of amenorrhoea, concluded that the tissue obtained was representative of the gonad as a whole, particularly with regard to its complement of germ cells, an opinion supported by some subsequent authors (Sykes and Ginsburg, 1971; Black and Govan, 1972; Zographos *et al.*, 1973; Egger, 1975; Fayez and Jonas, 1976; Motashaw *et al.*, 1977). Some (Zographos *et al.*, 1973; Egger, 1975) assumed one sample of at least 5 mm x 5 mm was necessary, while others argued that larger biopsies were required (Steele *et al.*, 1970; Sykes and Ginsburg, 1971)

Sutton (1974) was the first to seriously question the value of the ovarian biopsy and, based on his studies, he concluded at that time that it would be wrong to give the patient too grave a prognosis based on ovarian biopsy. He found that spontaneous pregnancies had occurred even in the complete absence of follicles within biopsy samples.

With advances in instrumentation and techniques, taking biopsies has become easier and more consistent. It remains, however, an invasive and, as such, hazardous, expensive and stressful operation.

It seems that the 1970s saw ovarian biopsy as an important advance in gynaecological diagnosis and prognosis, and since then it has become an issue of debate as to the reliability of these data (Khastgir *et al.*, 1994; Wallach, 1995). Lass *et al.* (1997) found that ovarian volume was not correlated with follicular density in women under

Table I. Number of follicles[a]

	Ovary A			Ovary B			Ovary C		
Age (years)	30			33			34		
Ovarian size (mm)	30 x 19 x 10			45 x 65 x 28			41 x 65 x 28		
Surface (mm^2)	1218			6648			5957		
In whole ovary[b]	30 907			2521			29 476		
Biopsy coordinates[c]	Counted in biopsy	Total contents predicted from biopsy	Percentage deviation from count of whole ovary	Counted in biopsy	Total contents predicted from biopsy	Percentage deviation from count of whole ovary	Counted in biopsy	Total contents predicted from biopsy	Percentage deviation from count of whole ovary
A1	95	36 860	+19	8	16 938	+572	92	174 536	+492
A2	20	7760	–75	13	27 524	+992	15	28 456	–3
A3	55	21 340	–30	5	10 586	+320	9	17 073	–42
A4	21	8148	–74	0	0	–100	32	57 709	+96
B1	46	17 848	–42	17	35 992	+1427	32	60 709	+106
B2	32	12 416	–60	0	0	–100	88	166 948	+466
B3	23	8924	–71	0	0	–100	36	68 296	+132
B4	8	3104	–90	6	12 703	+403	10	17 074	+42
C1	9	4392	–86	6	12 703	+403	10	18 972	–36
C2	257	99 716	+223	2	4234	+68	91	172 640	+486
C3	16	6208	–80	0	0	–100	9	17 074	+42
C4	2	776	–67	7	14 820	+488	0	136 433	+363
D	161	9982	–68	277	93 763	+3619	82	23 961	–19
Mean ± SD of all 2 mm biopsies		18 958 ± 27 289			11 292 ± 11 608			77 993 ± 65 700	

[a]Primordial follicles, primary follicles, 1–2 layer follicles, 2–3 layer follicles, multilayer follicles and antral follicles.
[b]Estimate based on quantitative morphological analysis of fully sectioned ovary plus those counted in the biopsies.
[c]See Materials and methods for explanation of coordinates; biopsies A–C were 2 mm in diameter; biopsy D was 5 mm in diameter.

35 years old but was highly correlated in women over 35 years old. They also found that follicular density decreases with advancing age. Women older than 35 had only a third of the concentration of follicles of younger women. FSH concentrations were not correlated with follicular density. Recently, Schmidt *et al.* (2003) showed that follicle density varied greatly in small pieces of cortex, rendering information from biopsies unreliable.

Is the lack of follicles in a single biopsy, or indeed any number of biopsies, enough to make accurate diagnoses or are they more misleading than prognosticating? If the data were reliable and could be used to counsel patients, then the risks associated with laparoscopic biopsy may be tolerable. We found that in individual cases, one or many more 'standard' biopsies could not reliably indicate the total numbers of follicles actually present in an ovary. In an attempt to estimate the number of follicles in the whole ovary, we converted biopsy information into total ovary information by transformation based on surface area. However, this approach did not yield acceptable results. One could argue that the surface area in the androgen-induced polycystic ovary (PCO)-like ovaries is enlarged and thus could affect the final predicted total using our method. The same would apply, however, to any method based on volume estimation. PCO is very common and, as such, the issue of estimating ovarian reserve also frequently applies. Every method in which biopsies are employed to predict a total need a translation via some morphometric characteristic. In ovaries, this is hazardous because their shape can rapidly change and is extremely dependent on their biological activity

From ovary A, which was most probably an entirely normal ovary, a number of biopsies had only a few follicles. This was a regular cycling 33-year-old woman who may well have been diagnosed with ovarian failure if these sparse biopsies had been taken, but who obviously had a fairly healthy store of follicles.

The quantity of oocytes in the biopsies was extremely variable even between closely related areas. This implies an inherent unreliability and unpredictability of individual biopsies.

In terms of absolute prediction, no region appeared to be more informative than any other. The mean of the 12 smaller biopsies proved no more accurate than individual biopsy data. The averages in ovary A and C are a reasonable assessment of reproductive potential. Whether women have ±20 000–70 000 follicles, as the estimate suggested, or ±30 000 as both the serial sections showed, they still seem able to reproduce. The average predicted contents in ovary B obviously overestimated the reproductive potential equivalent by some 10 years.

The original idea of the study was to take a maximum of 12 systematic small biopsies and investigate by computer randomization if a number of biopsies much less than 12 could be found which would approximate the true count and thus provide a clinically feasible procedure. However, since not even the average of 12 biopsies came close

and because of the immense inter-biopsy variation that was apparent, it was decided not to go ahead with this analysis.

Cysts, any large follicles or recent corpora lutea as well as abnormal ovarian shape could lead to sampling difficulties. These are inherent to the organ. We took the biopsies following removal of the ovaries, but even under these ideal circumstances, sampling and processing appeared highly susceptible to error.

The whole ovary sections were of sufficient distance apart to ensure that no follicle was counted twice. In the biopsies, however, occasionally, oocytes would appear in a series of sections, disappear and no nucleus became apparent. An attempt was made at following follicles from section to section to reduce the likelihood of double counting or of missing follicles with indistinct nuclei. The methods described and used for predictions and estimations throughout the current investigation rely on correction factors which are based upon assumptions regarding cell size, orientation and shape. However, none of the quantitative morphology techniques will overcome the inherent inaccuracy resulting from the unequal distribution.

Unequal distribution of follicles across the cortex of the ovary is the most obvious explanation for the great interbiopsy variation of numbers of follicles per mm^2 surface area. Our observations are in agreement with those recently published by Schmidt *et al.* (2003). They measured cortical follicle density per fragment volume in several entire ovaries and this varied from 0.007 to 166 follicles/mm^3.

There are several points in the above from which unequal distribution of oocytes may arise. The arrangement and growth pattern of the secondary sex cords into the ovary causes unequal dispersal of oocytes. The distribution of the follicles throughout the ovarian cortex from the stem cells is unlikely to be complete and will result in patches/nests of cells readily observable in the cortex. Blood supply has an influence and is an example of the fact that the ovary is an ever-changing organ. The presence of a corpus luteum will also affect the density. All these effects will contribute to the 'patchiness' of the follicular pattern and are likely to ensure that any biopsy taken will yield an inordinately large estimation if a nest is hit or a reserved estimation if they are missed.

An additional, important explanation for the lack of reliability of the follicle counts in the biopsy for predicting total counts is the fact that the very small numbers present in some specimens will give poor statistical precision in predicting the larger numbers because the impact of stochastic variation will be greater.

In conclusion, taking one or more ovarian biopsy does not seem to be the right procedure to estimate reliably the number of follicles in an ovary in the individual case.

This is largely the consequence of the unequal distribution of follicles across the surface of the ovary, in addition to technical shortcomings. Therefore, there is no place for this procedure in clinical evaluation of reproductive ageing. For research

purposes, it should only be used with caution and only for estimation of statistics for patients in groups whose sizes are such that they would compensate for the inherent extreme intra-individual and inter-individual spread of the parameter (Webber *et al.*, 2003).

Our negative experiences with this procedure are presented here in the context of the revival of interest in the subject and the subsequent debate. We hope that this contribution will add to the opinion that there is no place for the use of ovarian biopsy for the estimation of ovarian reserve.

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5 Estimation of follicle-stimulating hormone (FSH) threshold for initiating the final stages of follicular development in women with elevated FSH levels in the early follicular phase

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Abstract

Background: Women with regular menstrual cycles and elevated Follicle Stimulating Hormone (FSH) levels in the early follicular phase are believed to have diminished ovarian reserve. In assisted reproduction technologies (ART), this condition is associated with a low response to ovarian stimulation, a lower number of follicles, and lower pregnancy rates. A limited number of follicles which are available to respond to FSH (smaller cohort), or less sensitive follicles (higher FSH threshold) can be responsible for this. We tested the hypothesis that the follicle-stimulating hormone (FSH) threshold in patients with elevated FSH levels in the early follicular phase (EFP) is higher than in controls.

Methods: In six patients with elevated EFP FSH (> 10 IU/L) and 13 controls, the FSH threshold was determined by an ultra-low-dose step-up protocol with a GnRH agonist in the midluteal phase, before IV administration of recombinant FSH. The FSH threshold was determined by the mean of FSH levels of the above threshold value and the below threshold value.

Results: The FSH threshold in the elevated EFP FSH group was 6.75 IU/L and was significantly higher than the FSH threshold of the controls (4.65 IU/L). The FSH screening value on day 3 was 12.0 IU/L in the patient group and 5.0 IU/L in the controls. Estradiol was significantly lower on the day that the largest follicle was 18 mm in the elevated EFP FSH group compared with controls (277 vs. 491 pmol/L, respectively). On the day of hCG administration, the number of smaller (10-13 mm) follicles was equal but the number of larger (>14 mm) follicles was higher in the control group compared with the elevated FSH group. In the control group, the basal FSH levels correlated highly with the FSH threshold levels (r=0.8), but in the patients with elevated EFP FSH this correlation was absent.

Conclusions: In normal women, basal FSH day 3 values represent the ovarian threshold for FSH. In women with elevated day 3 FSH, the FSH threshold is higher but not as high as basal FSH values. We postulate that the FSH threshold in patients with elevated EFP FSH is higher because of intraovarian factors. Basal FSH overshoots the threshold, probably because of the limited feedback by the ovary.

Introduction

It is assumed that women who have regular menstrual cycles with elevated Follicle Stimulating Hormone (FSH) levels in the early follicular phase have diminished ovarian reserve (Mac Naughton *et al.*, 1992; Toner *et al.*, 1991). In assisted reproduction technologies (ART), this condition is associated with a low response to ovarian stimulation, a lower number of follicles, and lower pregnancy rates. A low response to FSH treatment in ART may indicate 1) that higher amounts of FSH are required to obtain a normal amount of follicles i.e., the follicles are less sensitive to FSH, and/or 2) that a limited number of follicles are available to respond to FSH (cohort). If the latter condition is true, then high FSH levels may indicate overshoot secretion by a lack of ovarian feedback. This would obscure possibly normal FSH sensitivity of the limited number of follicles.
The sensitivity of follicles for FSH can be expressed by the FSH threshold (Brown 1978; Schoemaker *et al.*, 1993; van der Meer *et al.*, 1994). The FSH threshold is the level that the plasma concentration of FSH must exceed to initiate the final stages of follicular development, i.e., from early antral (2-5 mm) to the preovulatory stage in the dominant follicle. The FSH threshold model has been intensively studied in patients with polycystic ovary syndrome (van der Meer *et al.*, 1994; van der Meer *et al.*, 1998; van Weissenbruch *et al.*, 1993) and in patients with hypogonadotropic amenorrhea (de Boer *et al.*, 1999). A special technique was developed to determine the FSH threshold during ovulation induction by van der Meer *et al.* (1994) from our group. We also used this technique to test the hypothesis that the FSH thresholds in patients with elevated early follicular phase FSH are higher than in regular cycling women with normal early follicular phase FSH concentrations. We determined the FSH thresholds in a group of women with elevated early follicular phase (EFP) FSH and controls.

Materials and Methods

Six patients with elevated EFP FSH, as defined by FSH levels on day 3 >10 IU/L in screening, regular menstrual cycles between 21 and 35 days, no experience of hot flushes, and no hormonal treatment in the last 3 months before the study, participated. Controls were 13 women recruited from the infertility population presenting with male subfertility or unexplained infertility. Controls had the same inclusion criteria except that the FSH levels on cycle day 3 needed to be < 10 IU/L. The median age was 33.5 (range, 26-39 years) in the control group and 32.5 (range, 27-39 years) in the elevated EFP FSH group. The median duration of infertility was 3 years (range, 2-5 years) in the control group and 2.75 years (range, 1.5-12 years) in the study

group. The median body mass index was 25.6 (range, 19.5-34.8) in controls and 22.6 (range, 20.3-36.2) in the EFP FSH group, which was not statistically significantly different.
Procedures followed in this study were in accordance with the Helsinki Declaration and with the guidelines of our institute. Informed consent was obtained from all participants.

Treatment protocol
Desensitization with a GnRH agonist (Triptorelin; Ferring, Hoofddorp, The Netherlands) 0.1 mg/day SC was started in the midluteal phase. Pituitary desensitization was confirmed by occurrence of menses, low LH, FSH and E_2 levels, and no follicles > 10 mm or cysts on ultrasound. After two weeks, IV administration of recombinant FSH (Gonal-F; Ares-Serono, Aubonne, Switserland) was started by the portable infusion pump (Auto syringe; Travenol Labs, Hooksett, NH). Follicular growth was monitored by transvaginal ultrasound using a 5-Mhz transducer (Ultramark 4; Advanced Technology Labs, Bothel, WA).
The FSH stimulation started with 37.5 IU (1/2 amp) daily, given in small doses every 30 minutes by the infusion pump. A stable level of serum FSH could be maintained in this way. This starting dose was continued for at least 7 days. If no ovarian response was observed (i.e., a growing follicle > 10 mm or an increase in E_2 > 200 pmol/L), the dose was increased with 1/4 amp steps (18.75 IU) every 5-7 days. Once a response was observed, the dose was kept constant until the largest follicle reached a diameter of 18 mm. To induce ovulation, 10.000 IU hCG (Profasi; Ares-Serono) was given IM. To support the luteal phase, 200 mg progesterone (Progestan; Organon, Oss, The Netherlands), three times daily was used intravaginally.

Assays
Blood samples were taken daily from the start of FSH stimulation until the day of hCG administration. Serum levels of FSH and E_2 were determined by commercially available assays (Amerlite; Amersham, UK), which were immunometric for FSH and competitive for E_2.
For FSH, the interassay coefficients of variation (CV) were 9% at 3 IU/L and 5% at 35 IU/L; the intra-assay CVs were 9% at 5 IU/L and 8% at 15 IU/L. The lower limit of detection for FSH was 0.5 IU/L (second International Reference Preparation 78/549). For E_2, the interassay CVs were 11% at 250 pmol/L and 8% at 3000 pmol/L; the intra-assay CVs were 11% at 350 pmol/L and 9% at 1100 pmol/L. The lower detection limit was 90 pmol/L.

FSH threshold
The FSH threshold level was determined as previously described by van der Meer *et*

al. (1994). In brief, the day at which follicle selection had taken place was determined in retrospect. Based on the assumption that a follicle starts FSH-dependent growth when its diameter is 5 mm (Chikazawa *et al.*, 1986) and that its growth rate is 2 mm/day (Ritchie 1985), the day at which follicle selection had presumably taken place was determined by retrograde extrapolation, counting backwards from the moment the dominant follicle had a diameter of 13 mm. The mean of the FSH level from this day, the day before, and the day after was defined as the above threshold level (ATV). The FSH level during the last 3 days of the preceding dose interval, at which no follicular growth had been observed, was defined as the below threshold value (BTV). The mean of the ATV and BTV was defined to be the threshold level and used for further calculations.

Statistics

The non-parametric Mann-Whitney *U*-test was used to compare FSH threshold, E_2, FSH screening values, follicle numbers, age and body mass index between the groups. Pearson's correlations between FSH thresholds and FSH screening values were calculated.

Results

The median (range) of FSH screening values (the day 3 FSH value in a spontaneous previous cycle) was 12.0 IU/L (11.0-14.0 IU/L) in the elevated EFP group and 5.0 IU/L (3.3-8.9 IU/L) in the control group ($P = 0.001$). The median (range) of the FSH threshold in the elevated EFP group was 6.75 IU/L (4.5-7.8 IU/L) and significantly higher ($P = 0.023$) compared with the control group median (range) of 4.65 IU/L (3.4-7.6 IU/L) (Fig.1). Follicle-stimulating hormone values 2 weeks after start of GnRH treatment but before stimulation with recombinant FSH were 3.55 IU/L (2.0-5.5 IU/L) in the elevated FSH group and 2.68 IU/L (1.9-3.4 IU/L) in the control group, which was not significantly different. The median (range) total number of FSH ampules used was not different between the groups: 13 ampules (8-29 ampules) in the elevated EFP group and 16 ampules (10-41 ampules) in the control group. In the control group, the basal FSH concentration correlated significantly with the threshold values for FSH ($r = 0.8$; $P < 0.01$) (Fig. 2). This correlation became less when calculated for the whole group ($r = 0.69$; $P = 0.01$) and was absent in the elevated EFP group.

Follicles

The total number of follicles was compared at the time the largest follicle was 18 mm in diameter. The number of smaller follicles (10-13 mm diameter) was not different between the groups (median 1, range 0-3 for both groups), but the number of larger

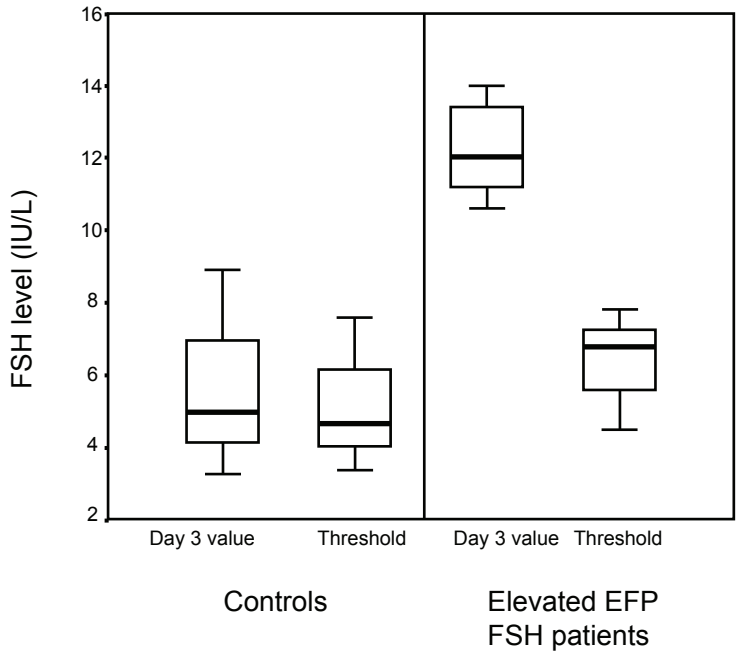

Figure 1. Box-and-whisker plots of the FSH day 3 values and FSH thresholds in the elevated EFP group and the control group. The center bar of each box represents the median; the top and the bottom of each box represents the 75th and 25th percentiles

follicles (14-18 mm) was higher in the control group with median (range) of 2 (1-4) vs. 1 (1-2) in the elevated EFP patients ($P < 0.05$).

Estradiol

Median (range) E_2 levels on the day of hCG administration was significantly lower in the elevated EFP group than in the control group: 277 pmol/L (257-354 pmol/L) versus 491 pmol/L (220-1620 pmol/L) ($P = 0.027$).The E_2 levels on the day of hCG administration correlated with the number of follicles of 14-18 mm in diameter ($r = 0.78$, $P = 0.001$).

Discussion

This study shows that in women with early follicular phase FSH concentrations in the normal range, the day 3 FSH level represents the FSH threshold. The median FSH day 3 values and the FSH thresholds in the controls were similar and showed a high correlation ($r = 0.8$; $P < 0.01$). This means that in women with normal day

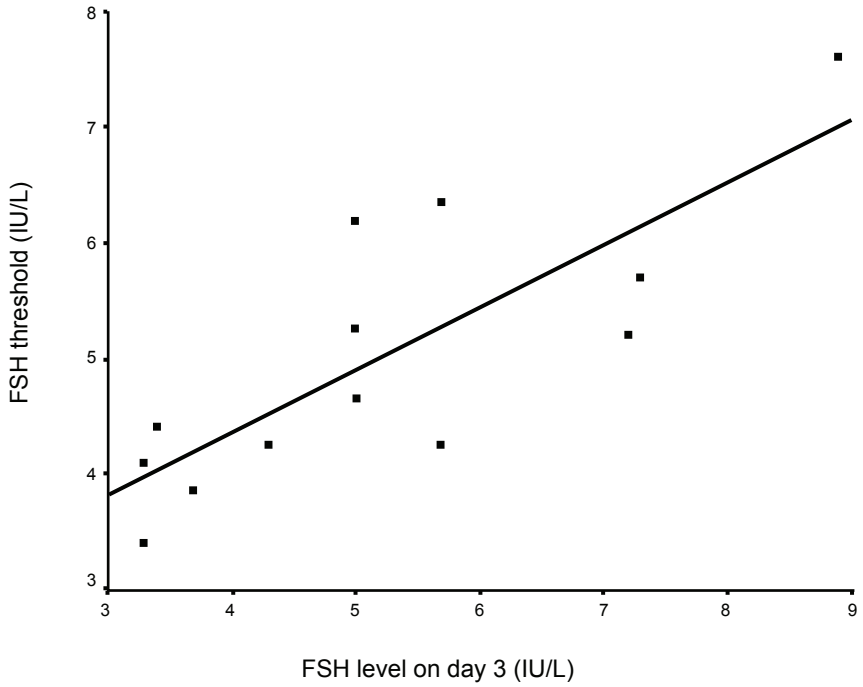

Figure 2. Correlation between basal FSH concentration and FSH threshold values in the control group. r = 0.8; $P < 0.01$

3 FSH concentrations, interindividual variations in FSH levels represent individual differences of follicle sensitivity for FSH. This was also suggested by other investigators (Schipper *et al.*, 1998) in a group of normal women.

In women with elevated early follicular phase FSH the threshold is significantly higher than in controls. Nevertheless, this increase is modest compared with the day 3 FSH values found in these patients. Apparently, in this situation, in contrast to normal women, the EFP FSH concentration does not represent the threshold but is much higher.

Our observation of the elevated threshold strongly suggests that intraovarian factors are involved. One explanation would be that the FSH receptors are less sensitive to FSH. One study (Perez Mayorga *et al.*, 2000) showed that a certain polymorphism of the human FSH receptor gene resulting in substitution of asparagine by serine at position 680 and threonine by alanine at position 307 of the protein results in a less sensitive receptor. In IVF patients, this genetic variant was found to be homozygous in 26% and heterozygous in 45% of cases. Women with this variant had significantly higher FSH concentrations than those without this variant in the early follicular phase

of the menstrual cycle, and they required significantly more FSH during stimulation of the IVF procedure to obtain the same number of follicles. Therefore, the elevated early follicular FSH in our patients may represent this FSH receptor genotype, which implies that it is part of natural variation without implications for fecundity. This notion is supported by another observation that subfertility patients with elevated FSH who were followed up for 3 years had a 50% chance to deliver a baby, which was the same as those without elevated FSH (van Montfrans *et al.*, 2000).

Intraovarian conditions that determine the response of the ovary to FSH may be involved. A number of intraovarian factors have been published in that context, among which are IgF-I, GH, inhibins and activins (Oosterhuis *et al.*, 1998; Klein *et al.*, 2000; Owen *et al.*, 1991; Woodruff *et al.*, 1990; Klein *et al.*, 1996; Santoro *et al.*, 1999; Welt *et al.*, 1999; de Koning *et al.*, 2000).

It is remarkable that according to our findings the actual elevated early follicular phase FSH levels in our patients are much higher than the elevated FSH threshold. Apparently at that time of the menstrual cycle an overshoot of FSH is secreted by the pituitary because of limitations of ovarian feedback. This means that the actual FSH represents a composite of 1) a slightly higher FSH threshold and 2) a surplus of FSH secretion due to a lack of ovarian feedback.

The elevated FSH threshold in patients with elevated early follicular phase FSH suggests that there is some rationale in administering higher dosages of FSH in patients with elevated basal FSH. This would only apply for situations in which endogenous FSH secretion is completely suppressed, such as by a GnRH agonist. Under unsuppressed ART conditions such as natural IVF or in the early phase of GnRH antagonist IVF cycles, an endogenous surplus FSH secretion (median basal FSH of 12 IU vs threshold of 6.75 IU) is already present. It should be strongly questioned whether under unsuppressed circumstances a further addition of FSH will lead to more oocytes and embryos and ultimately more pregnancies in these patients.

In summary, we have shown that 1) early follicular phase FSH concentrations represent the ovarian threshold for FSH in normal patients and 2) elevated early follicular phase FSH concentrations represent a composite of a higher threshold and overshoot secretion. Intraovarian factors such as FSH receptor polymorphisms and autocrine and paracrine action of compounds such as inhibins, activins and IgF may be involved.

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6 Falsely elevated Follicle-Stimulating Hormone levels in women with regular menstrual cycles due to interference in immunometric assay

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Abstract

Background: Elevated FSH levels in the early follicular phase are used to counsel patients in assisted reproduction because it can indicate diminished ovarian reserve.

Material and methods: We describe three cases with elevated FSH levels which were not related to diminished ovarian reserve but interference in the immunoassay.

Results: After serial dilution the FSH levels were much lower than expected and after PEG precipitation FSH values became normal.

Conclusion: Elevated FSH values can be caused by disturbances in the immunoassay. Serial dilution and PEG precipitation can reveal such interferences.

Introduction

In assisted reproduction programs, it becomes routine to screen women on cycle day 2-4 to be informed about their prognosis in controlled ovarian hyperstimulation. An elevated follicle-stimulating hormone (FSH) level on day 3 correlates well with poor response to exogenous gonadotropins (Toner *et al.*, 1991; Scott and Hofmann 1995). In many clinics, day 3 FSH values are already used to make clinical decisions for infertility treatment. In most laboratories, FSH concentrations in serum are measured by competitive immunoassays such as radioimmunoassay (RIA) or more recently by two-site immunometric assays such as immunoradiometric assays. Falsely elevated serum FSH concentrations measured with RIAs have been reported in women with a history of vaccination with inactivated bactera cultured in rabbit tissue (Padova *et al.*, 1991) and in a patient who had worked with rabbit serum (Cahill *et al.*, 1992). Also, interference for immunoradiometric assays has been described, but to our knowledge not for FSH (Taylor and Khoury 1997).
We report three women with falsely elevated serum FSH levels measured by immunometric sandwich assay. Their FSH levels were in the peri- and postmenopausal range. We verified the FSH values by serial dilution and by precipitation of possible interfering high-molecular substances with polyethylene glycol (PEG).

Material and Methods

Three subjects with spuriously elevated FSH concentrations are described. Patients characteristics are given in Table 1.
Patient 1 was 30 years old, para 0, with regular menstrual cycles, and no history of infertility; she was asked to participate in a study as a control. Cycle analysis showed a biphasic basal body temperature (BBT). Cycle day 3 (CD 3) FSH was 44 IU/liter; CD3 estradiol and luteinizing hormone (LH) levels were normal (105 pmol/liter and 3.2 IU/liter, respectively), and midluteal progesterone level was normal (88 nmol/liter). Spontaneous pregnancy occurred as soon as child wish was present.
Patient 2 was 37 years old, para I, secondary infertility. Cycle analysis revealed an ovulatory cycle with biphasic BBT and ultrasound monitored ovulation. CD3 FSH was 60 IU/liter. Normal LH, estradiol, and midluteal progesterone levels were found (3.6 IU/liter, 91 pmol/liter, and 92 nmol/liter, respectively). Three years later she conceived spontaneously.
Patient 3 was 29 years old, para 0, regular menstrual cycles, primary infertility of 3 years. Day 3 FSH in screening was 15 IU/liter. Cycle analysis showed biphasic BBT, ultrasound-monitored ovulation and normal LH, estradiol and luteal progesterone levels (5.5 IU/liter, 156 pmol/liter, and 110 nmol/liter, respectively). In two controlled ovarian hyperstimulation cycles 9 and 10 oocytes, respectively, were obtained for in

Table 1. Characteristics of patients showing falsely elevated FSH

	Patient 1	Patient 2	Patient 3
Age (years)	30	37	29
CD3 FSH (U/liter) [a]	44	60	15
CD3 LH (U/liter)	3.2	3.6	5.5
CD3 E_2 (pmol/liter)	105	91	156
Midluteal progesterone (nmol/liter)	88	92	110
Ultrasound	ovulation	ovulation	ovulation
Infertility	No infertility	Idiopatic	Male factor

[a] CD3, cycle day 3

Table 2. FSH results from serial dilutions and PEG precipitation of serum

	Calculated results of FSH (U/l) after serial dilution or after PEG precipitation of serum		
Sample analysed	Patient 1	Patient 2	Patient 3
100% serum	44	60	15
50% serum	18	28	9.7
12.5% serum	10	21	6.3
PEG-precipitated serum	4	5.5	5.2

vitro fertilization. This was an unexpected good response. She became pregnant in the second treatment cycle.

FSH was measured both in undiluted and in diluted serum samples from the three subjects. Serial dilution of serum samples was made in the zero matrix of the assay. PEG precipitation was done by mixing equal amounts of serum and ice-cold solution of 30% (wt/vol) polyethylene glycol-600. Treatment with PEG precipitates endogenous immunoglobulines, including heterophilic antibodies. After centrifugation, FSH was measured in the supernatant. For preparation of the calibration curve, the calibrators were treated likewise with PEG.

Results

After dilution the FSH values found were much lower than expected values; also, nonlinearity was found (Table 2). After PEG precipitation, the FSH values measured in the supernatant were in the normal range. The process of dilution or PEG precipitation did not cause a change in measured FSH values in serum from control subjects with normal FSH values.

Discussion

In clinical infertility practice, decisions are made on the basis of one or two laboratory findings with respect to elevated serum FSH. When a patient shows elevated FSH values in the early follicular phase, concern is raised about the ovarian capacity. We here describe three women with elevated serum FSH levels, but with normal ovarian function. In these cases the FSH values appeared to be falsely elevated, as shown by the results of serial dilution and PEG precipitation tests. A nonlinear dilution test points to the presence of substances that behave in the assay in a way different from the FSH standard. This test, which can be easily performed, can be used as a first indication that interference in the assay is present. With PEG precipitation, interference from circulating high-molecular proteins can be prevented. The results from the PEG tests show that the interference has been removed and might be immunoglobulins, probably heterophilic antibodies. These heterophilic antibodies can cross-link the detecting labeled antibody to the captivating antibody, thus giving artifactually high results (Levinson 1997). Both tests, serial dilution and PEG precipitation, can be easily done in most laboratories. However, for the PEG precipitation test a separate PEG run is necessary because all calibrators have to be PEG-treated.

We recommend clinicians who use day 3 FSH values in regular cycling women to screen for ovarian incompetency, to ask the laboratory for reanalysis of samples with FSH values above the cutoff value for medical decision. Any discrepant value after dilution of the sample can indicate whether further steps, e.g., PEG precipitation, cross-check with an alternative assay system, or measurement of additional hormones, are justified.

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7 The distribution of FSH receptor isoforms is related to basal FSH levels in subfertile women with a normal menstrual cycle

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Abstract

Background: Recently a polymorphic variant of the FSH receptor in which amino acid asparagine (Asn) on position 680 is replaced by serine (Ser) was found. This is associated with higher FSH levels in the early follicular phase and an increased FSH requirement to obtain follicular response in IVF patients. The aim of our study was to test the hypothesis that this receptor isoform occurs more often in regularly menstruating subfertility patients with elevated basal FSH.

Methods: A retrospective cohort study of 38 subfertility patients with a regular menstrual cycle and elevated FSH (FSH>10 IU/L) compared to 40 patients with normal early follicular phase FSH was carried out. DNA was analysed to determine the FSH receptor genotype.

Results: The N680S variant on one or both alleles of the FSH receptor gene was significantly more prevalent in patients with elevated FSH ($P< 0.05$). The homozygous Asn/Asn variant at codon 680 was found in 45% of women with normal FSH and in 21% of women with elevated FSH. The homozygous Ser/Ser receptor variant was present in 12.5 % of women with normal FSH and in 21 % of patients with elevated FSH. Also the heterozygous combination of both variants Asn/Ser occurred more often in women with elevated FSH (58 % vs. 42.5 %).

Conclusions: The N680S sequence variation of the FSH receptor is found in >75 % of the cases with elevated basal FSH and suggests a higher FSH threshold.

Introduction

It has been known for many years that raised FSH levels in the early follicular phase of the menstrual cycle are present in normal older women (Sherman and Korenman, 1975) as well as in subfertile older women (Ahmed Ebbiary *et al.*, 1994, Lambalk, 1998a). Many studies have indicated that elevated basal FSH concentrations in the early follicular phase have a strong negative impact on pregnancy outcome in assisted reproduction (Scott *et al.*, 1989; Toner *et al.*, 1991). Some reports suggest that a distinction should be made between younger and older patients with elevated FSH in the early follicular phase (Check *et al.*, 1998; van Rooij *et al.*, 2003). It seems, however, that the predictive value of basal FSH in the general subfertility patient is of much less value and unable, even with high threshold values, to distinguish clearly between those patients who will have a baby and those who will not (van Montfrans *et al.*, 2000; van Rooij *et al.*, 2004).

The monotropic rise of FSH in association with ageing is the result of a decline in ovarian hormonal feedback, in particular that of inhibin B (Welt *et al.*, 1999; Klein *et al.*, 2004). Also in younger subfertility patients with elevated FSH, lower inhibin levels are found, indicating limited ovarian function (de Koning *et al.*, 2000a). This limitation is the result of a quantitative and qualitative demise of available follicles. In subfertility patients with elevated FSH it has been shown that the threshold for FSH of the follicle is slightly increased (de Koning *et al.*, 2004) which suggests that the ovary is less sensitive to FSH. Theoretically such patients may have FSH receptors which are less sensitive to FSH.

Recently a polymorphic variant of the FSH receptor was found in which the amino acid asparagine (Asn) at position 680 is replaced by serine (Ser) (N680S). The N680S variant was associated with higher FSH levels in the follicular phase starting from luteal-follicular transition (Greb *et al.*, 2005) and more FSH was needed to obtain normal follicular response in IVF patients (Perez Mayorga *et al.*, 2000; Sudo et al, 2002). The latter findings suggest that this receptor variant is less sensitive to FSH and that higher endogenous FSH levels may represent a natural compensation which is needed to enable normal follicle growth. In a group of normogonadotropic anovulatory women, the homozygous N680S variant was found to be more prevalent with higher basal FSH levels. (Laven *et al.*, 2003) So far, *in-vitro* experiments could not show differences in activity of the various allelic variants of the FSH receptor (Simoni *et al.*, 1999; Sudo *et al.*, 2002). FSH receptor sequence variation does not play a role in complete ovarian failure (Conway *et al.*, 1999).

The aim of the present study was to test the hypothesis that in regularly menstruating subfertility patients with elevated basal FSH the N680S variant of the FSH receptor occurs more often. We investigated the distribution of these polymorphisms in a group of ovulatory subfertility patients with and without elevated FSH.

Materials and methods

The current study was approved by the Institutional Review Board and written, informed consent was obtained from all participants. For the purpose of this study we approached patients that had participated in an earlier study. The original investigation was a nested case-control study in 50 consecutive patients diagnosed as having a diminished ovarian reserve according to elevated basal FSH concentrations (>10.0 IU/l), and in 50 controls. This was a questionnaire study that investigated occurrence of pregnancy (van Montfrans *et al.*, 2000). From this study cohort, 38 patients with basal FSH > 10.0 IU/l and 40 controls gave written, informed consent allowing us to sample blood for FSH receptor DNA analysis. In the patient group, 36 women were Caucasian and two Asian, and in the control group all women were Caucasian.

Original patient cohort and control group
Basal FSH concentrations, taken on day 2,3 or 4 of the menstrual cycle, were measured routinely in all newly registered subfertility patients in our department from January 1, 1995. Using a computerized database of patients registered after this date, we identified a cohort of 50 consecutive patients with elevated basal FSH concentrations (>10.0 IU/l) that fulfilled all inclusion and exclusion criteria. Inclusion criteria were a basal FSH concentration >10.0 IU/l, maximum age at registration 40 years, subfertility of ≥ 12 months and ovulatory menstrual cycles (assessed by basal body temperature chart, serum progesterone levels in the luteal phase or by a luteal phase endometrial biopsy). Patients with a history of unilateral ovariectomy, chemotherapy or irradiation were excluded. Eleven patients with basal FSH concentrations >10.0 IU/l were not included in the study for the following reasons: age at registration >40 years (n = 4), history of unilateral ovariectomy (n = 6) or a history of chemotherapy (n = 1). In the same database we identified the first 50 age-matched controls with basal FSH concentrations < 10.0 IU/l and concurrent estradiol concentrations <200 pmol/l. Other inclusion and exclusion criteria were the same as in the study group.

Laboratory assays
Basal FSH concentrations were measured with an immunometric assay (Amerlite, Amersham, UK). The assay was calibrated against the second International Reference Preparation for FSH (78/549). The intra- and inter-assay coefficient of variation were 9 and 8% for FSH values <25 IU/l, respectively. Estradiol concentrations were measured with a competitive immunoassay (Amerlite, Amersham, UK), with intra- and inter-assay coefficient of variation of 13 and 11% respectively for estradiol concentrations <500 pmol/l. All samples were run in duplicate.

DNA analysis

Genomic DNA was isolated from EDTA blood samples using the FlexiGene DNA extraction kit (Qiagen, Germany). PCR amplification of the two DNA portions containing SNP at nucleotide positions 919 and 2038 within exon 10 of the FSHR gene were performed according to Gromoll *et al.* (2000). The detection of the SNP distribution was conducted using an allelic discrimination assay based on TaqMan technology (Simoni *et al.*, 2002). Homozygous controls for each of the polymorphisms were included in each run.

Statistical analysis

Statistical tests were performed using SPSS Base 7.5 for Windows 9 (SPSS inc., Chicago, IL, USA). We performed analysis of variance and Student's t-test, linear-by-linear association analysis and χ^2 tests when appropriate. Values for age, FSH, estradiol and cycle length were normally distributed after logarithmic transformation. Statistical significance was set at $P<0.05$.

Results

Baseline characteristics are given in Table 1. Statistically significant differences between the groups with elevated and normal basal FSH concentrations were noted for menstrual cycle length and for day 3 FSH concentrations.

There was a significantly larger number of patients with elevated FSH that had the N680S variant on one or both alleles of the FSH receptor gene ($P< 0.05$). The homozygous Asn/Asn receptor type was found in 45% of women with normal FSH and only in 21% of women with elevated FSH whereas the homozygous Ser/Ser state was present only in 12.5 % of women with normal FSH and in 21 % of patients with elevated FSH. Also the heterozygous Asn/Ser state occurred more often in women with elevated FSH (Table 2). This distribution was significantly different ($P< 0.05$).

The length of follow-up with regard to take home baby rate was 7.5 years (follow-up: period between the moment the couple attempted to achieve a pregnancy and the most recent patient contact). Of the women with elevated FSH, 37.5% with homozygous Asn/Asn FSH receptor had a baby while this was the case in 59% and 50 % women with respectively heterozygous or homozygous N680S variant (by linear association $P = 0.8$) (Table 3). In the control group 50 % of women with Asn/Asn, 58 % of those with Ser/Asn and 40 % of women with Ser/Ser FSH receptor had an ongoing pregnancy. No significant differences with regard to take home baby rate in relation to FSH receptor polymorphism was seen.

In the elevated FSH group, 12 women conceived naturally and eight pregnancies were achieved after assisted reproduction treatment (60 and 40 % respectively,

Table I. Baseline characteristics of subfertility patients with elevated and normal FSH

	FSH > 10 IU/l	FSH < 10 IU/l	*P*
n	38	40	
Age (years)	34 ± 4.2	33.8 ± 3.9	ns
FSH (day 3) (IU/l)	15.3 ± 4.8	5.8 ± 9.0	< 0.0001
Estradiol (pmol/l)	110 ± 64	118 ± 35	ns
Cycle length (days)	26.7 ± 2.5	28.5 ±2.6	< 0.001

ns = not significant

Table II. Distribution of FSH receptor genotypes (codon 680) in patients with and without elevated FSH*

	Asn/Asn (*n*=26)	Asn/Ser (*n*=39)	Ser/Ser (*n*=13)
FSH > 10 IU/l (*n*=38)	8 (21 %)	22 (58 %)	8 (21 %)
FSH < 10 IU/l (*n*=40)	18 (45 %)	17 (42.5 %)	5 (12.5 %)

*: $P < 0.05$ (linear-by-linear association)

Table III. Take home baby rate in patients with elevated FSH according to FSH receptor genotype (codon 680)

Asn/Asn	Ser/Asn	Ser/Ser
3/8 (37.5 %)	13/22 (59 %)	4/8 (50 %)

$P = 0.8$ (linear-by-linear association)

not significant). Women with normal basal FSH values had naturally conceived pregnancies in 52 % of cases and 48 % after assisted reproduction treatment.

Discussion

This study confirms our hypothesis that in subfertility patients with elevated FSH and regular menstrual cycles, the N680S FSH receptor sequence variation, either on one or both alleles, occurs more frequently than in patients with normal FSH. The distribution of the FSH receptor genotypes among the women with normal FSH agrees with that described in ovulating Japanese women (Sudo *et al*., 2002). In the present study the occurrence of the Asn/Asn FSH receptor genotype in 45% in the women with normal FSH was higher compared to the 29-31 % found in earlier studies in assisted reproduction treatment patients or healthy controls (Perez Mayorga *et al*., 2000; Laven *et al*., 2003; Daelemans *et al*., 2004). The most likely explanation

for this difference is that the patient population in the other studies was a random mixture of IVF patients or controls with normal and potentially elevated FSH, which was not the case in the control group of the current study.

It seems that the Ser/Ser and Ser/Asn genotypes occur very frequently in the various populations without fertility disorders and it is therefore most likely not a marker for a certain type of reproductive failure. It is nevertheless remarkably associated with higher FSH levels. Assisted reproduction treatment patients with these receptor variants, undergoing stimulation with FSH for induction of multiple follicle growth, required higher dosages in order to obtain a normal response (Perez Mayorga *et al*., 2000). A retrospective study in IVF patients has shown an association between the presence of Ser at codon 680 and poor responses to gonadotropin stimulation (de Castro *et al*., 2003). Recently, a prospective study showed a lower estradiol response in IVF stimulation in women with the Ser/Ser genotype, compared to women with Asn/Asn with the same gonadotrophin dose (Behre *et al*., 2005). Such clinical observations suggest that the N680S allele is associated with a receptor protein slightly less sensitive to FSH. Subsequently, an altered set point of ovarian feedback mechanisms provides the higher FSH as a natural compensation allowing normal follicle growth.

Currently, measuring basal FSH is a routine procedure during the diagnostic work-up of the subfertile couple for prognostic evaluation and to predict ovarian response to gonadotropin stimulation (Kwee *et al*., 2003).

In our clinic, elevated FSH (>8 IU/l) occurs in about 2.5 % of the female patient population (Lambalk *et al*., 1998a). We think that in at least a number of these subfertility patients, particularly in the presence of a regular ovulatory cycle, elevated FSH is not an indication of limited ovarian reserve but rather a representation of a different FSH receptor genotype.

Should we now perform a genotype analysis in all patients with an elevated FSH? It is too early for such a conclusion based on the current study. It seems, however, worthwhile to evaluate in a prospective study the contribution of FSH receptor polymorphisms in IVF stimulation in relation to gonadotropin stimulation dose, and in terms of number of live births.

The notion that a patient has elevated FSH as a result of a less sensitive receptor may have practical implications when FSH stimulation is needed to obtain multiple follicle development. In IVF stimulations an *a priori* higher FSH starting dose with FSH would be logically justified. Another implication seems to be the grade of severity of ovarian hyperstimulation syndrome (OHSS) that may occur upon standard ovarian stimulation in IVF (Daelemans *et al*., 2004).

In patients with a less sensitive FSH receptor, elevated FSH is not a good parameter for fecundity prognosis. Indeed, recently several studies have indicated that basal FSH in the general subfertility patient with a regular menstrual cycle is of limited

value to predict ongoing pregnancy (van Montfrans *et al*., 2000; van Rooij *et al*., 2004).

Theoretically it could be expected that patients with the Asn/Asn receptor and elevated FSH are more likely to be close to imminent ovarian failure. Nevertheless, it appeared from our study that about one-third of these women subsequently delivered a child. This was not significantly different compared to the women in the other groups. It is likely that in this matter the limited size of the groups excludes any statistically justified outcome.

It should be realized that there are also other causes of elevated FSH that are not associated with FSH receptor polymorphisms or imminent ovarian failure (Lambalk, 2003). Hereditary dizygotic twinning is associated with elevated early follicular phase FSH concentrations (Lambalk *et al*., 1998b). However, a negative logarithm of the odds (LOD) score for markers at the locus of the FSH receptor in a large sib pair study of mothers with dizygotic twins, makes involvement of the FSH receptor in this natural condition unlikely (Lambalk 2001, Montgomery *et al*., 2001). Furthermore, heterophilic antibodies disturbing the FSH assay may be responsible for spuriously elevated FSH (de Koning *et al*., 2000b). This was ruled out in all patients that had participated in the current study.

In conclusion, the N680S allele of the FSH receptor is indeed associated with elevated basal FSH in >75 % of the cases and suggests a higher FSH threshold. From this study we cannot conclude that patients with Asn/Asn FSH receptor and elevated FSH have a less favourable chance of pregnancy. However, with the interpretation of elevated early follicular phase FSH in infertility patients with a regular menstrual cycle, we have to consider that it may represent a frequently occurring isoform of the FSH receptor.

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8

General discussion

Since the mid eighties measurement of early follicular phase FSH has become a worldwide routine practice in the endocrine workup of the female infertility patient. An elevated FSH value relates to poor response in assisted reproduction, supposedly as a result of limitation of the ovarian reserve. In older perimenopausal women critical depletion of ovarian oocyte content invariably associates with higher FSH levels. Over the past two decades extensive knowledge resulted from many studies detailing the reproductive endocrinology in association with reproductive aging. In most of these studies women of advanced age were studied and the results were simply extrapolated to substantially younger women who were clinically evaluated for infertility, with the assumption that the elevated FSH is a signal for protracted ovarian aging. So far, detailed description of the reproductive endocrinology in these younger women was not available.
In this thesis we described the detailed endocrinology of relatively younger women with a history of elevated day 3 FSH levels. This was evaluated not only at the level of the ovary but also at the pituitary level by evaluating the pulsatile pattern of pituitary LH and FSH release in women with a history of elevated early follicular phase FSH compared with controls. We also described some other causes of elevated FSH levels which are likely not related to diminished ovarian reserve.

Endocrine profiles throughout the cycle

By studying the day to day values of FSH, LH, oestradiol, progesterone and inhibins we found that in women with a history of elevated FSH in the early follicular phase and again in the cycle we studied, the endocrinology resembles that of older women as described in other studies: serum FSH is elevated throughout all phases of the cycle together with lower inhibin B levels in the follicular phase, during the LH surge, and again in the late luteal phase. Inhibin A is also lower in the early follicular phase. Therefore also in these younger women the elevated FSH reflects diminished ovarian inhibin feedback from a smaller available cohort of follicles in relation to limited oocyte reserve. This is confirmed by a low level of AMH in the early follicular phase (Figure 1).
In our study, women with a history of elevated early follicular phase FSH, but a normal FSH level on cycle day 3 in the cycle we monitored, we found the same low level of AMH compared with the group with consistently elevated FSH, indicating that they should be regarded as having a limited ovarian reserve. When FSH turns to almost normal in these women, most reproductive hormones became comparable with controls. However, in the peri-ovulatory phase in these women elevated inhibin A levels were found, which was also noted by others (Klein *et al.*, 1996). Probably multiple follicle growth and thus more granulosa cells which are stimulated by slightly higher FSH levels are responsible for this (Figure 2).

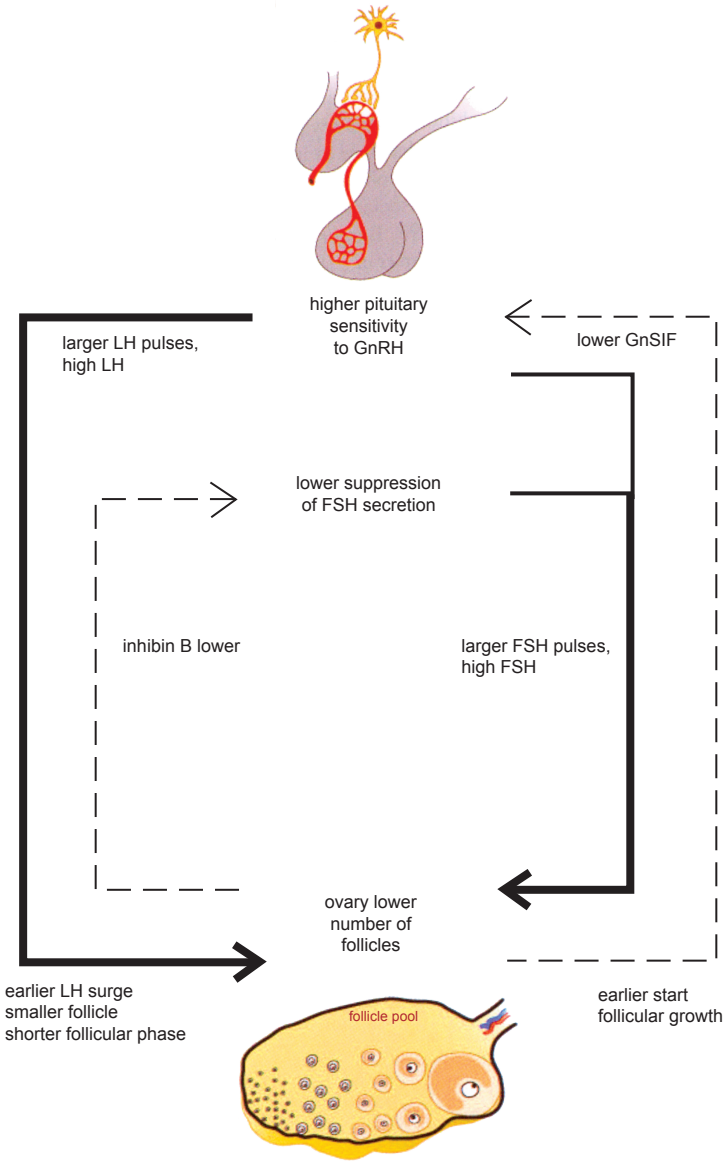

Figure 1. Endocrinology and follicular growth in women with consistently elevated FSH

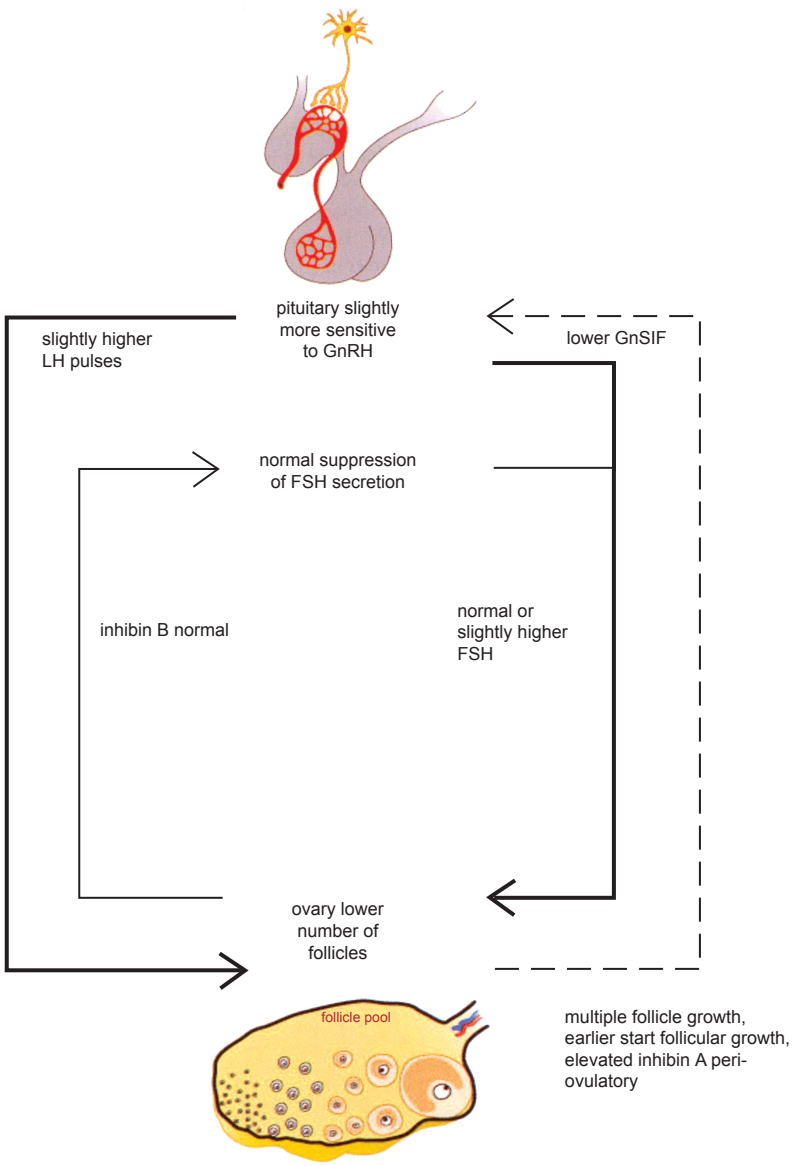

Figure 2. Endocrinology and follicular growth in women with variably elevated FSH in a cycle when basal FSH is normal.

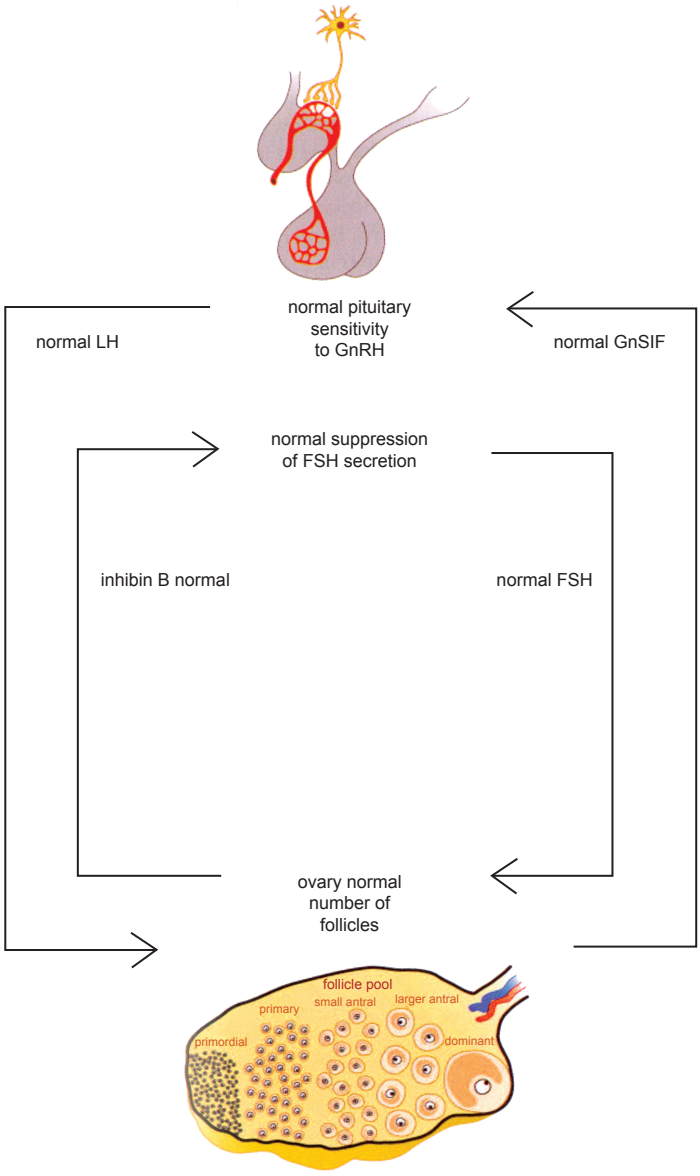

Figure 3. Normal endocrinology in controls.

Pituitary episodic secretion and responsiveness of gonadotrophins in women with elevated FSH

The higher FSH levels are associated with secretion of larger FSH pulses. This is likely the result of increased responsiveness to GnRH as we showed in our studies. Thus to some extend the monotropic rise of FSH is GnRH dependent. On the other hand it is known that FSH secretion that can be suppressed by inhibins is mainly a GnRH independent component. Our studies and many others indicate that a decreased inhibin tone is associated with diminished ovarian reserve with the rise in FSH as a consequence. We believe that superimposed on this mechanism additional factors that govern pituitary responsiveness to GnRH play a role. Since in our studies LH response to GnRH is also increased whereas oestradiol levels were unaltered, we suggest this increased sensitivity of the pituitary to GnRH is possibly due to lower ovarian secretion of the putative Gonadotrophin Surge Inhibiting Factor (GnSIF). GnSIF is secreted by granulosa cells and keeps the pituitary in a low state of responsiveness to GnRH. This hypothesis is supported by recent observations of decreased GnSIF bioactivity in the natural cycle of women with poor response in IVF (Martinez *et al.*, 2002).
Summarizing: elevation of gonadotropic hormones in women with elevated basal FSH is the net result of relaxation of 1) ovarian inhibin restraint of GnRH-independent pituitary FSH secretion, and 2) relaxation of the restraint by GnSIF on GnRH-dependent FSH and LH secretion rather than changes in ovarian steroid hormones.

Intercycle variability related to elevated FSH

An important aim of our work was to better understand the phenomenon of day 3 FSH values fluctuating from cycle to cycle. It seems that two distinct mechanisms may underlie this.

1. Early follicle development has already started in the luteal phase of the preceding cycle. Consequently dominance is reached earlier, accompanied with substantial oestradiol secretion which causes negative feedback and lowers FSH. Indeed often lower FSH values seen in patients with previously elevated FSH are observed together with higher E_2 levels. As such higher early follicular phase E_2 levels are considered as an indication of limited ovarian reserve (Licciardi *et al.*, 1995; Smotrich *et al.*, 1995; Evers *et al.*, 1998). This protracted follicular development also to some extend explains the often noted shortening of the follicular phase (aside from the smaller maximal diameter at time of ovulation).
In our evaluation we did not note elevated E_2 levels in those patients with normalized

FSH, nor did we find a shorter length of the follicular phase. A likely explanation for this is that our study design demanded a minimal cycle length of 21 days for inclusion. Therefore we probably excluded women with elevated E_2 levels caused by advanced follicular growth and shorter cycles from our analysis. This initially unintended exclusion may in the end have allowed us to detect another mechanism contributing to temporary normalization of FSH.

2. We showed for the first time that a normal day 3 FSH level in women with variable elevated early follicular phase FSH levels, is associated with normal levels of inhibin B in the preceding luteal phase (chapter 2). When day 3 FSH levels were high, decreased inhibin B levels were found in the preceding luteal phase. Since inhibin B is related to the cohort of follicles which become FSH dependent, a high day 3 FSH may reflect a relatively smaller available cohort in that cycle. The question is if this notion may be of some benefit for the clinical approach towards these patients in particular with IVF. Based on our finding, that suggests presence of a temporary larger cohort when basal FSH normalizes, one would indeed expect a better ovarian response upon hormonal hyperstimulation as suggested earlier by Lass (Lass *et al.*, 2000). The only study published in this respect (Abdalla and Thum, 2006) found no differences. In their retrospective study however, samples used for the estimation of the FSH were often not collected in the beginning of the same IVF stimulation cycle from which outcome was measured but from a preceding cycle. Our study indicates that in these types of patients intercycle variability of FSH is a common feature, which means that if a basal FSH value is to be used to determine the actual size of the cohort, it should be a value in the early follicular phase of the stimulation cycle. A prospective trial with uniform prevention of premature luteinization (short protocol GnRH agonist or a GnRH antagonist) and standard gonadotrophin stimulation would establish or rule out the possible usefulness of such a strategy.
In conclusion, variation of day 3 FSH levels can be explained by:

1. advanced follicular growth due to an earlier start of the growing follicle, which results in a shorter cycle length.
2. variation of cohort size with temporary normalization of the cohort.

Multiple follicle growth related to elevated FSH

The relation between elevated FSH levels and multiple follicle growth has been discussed since the relation between aging and dizygotic twinning was established (Bulmer, 1970). Increased levels of FSH were found in mothers of dizygotic twins (Lambalk *et al.*, 1998) with normal ovarian feedback of steroids and inhibins. Also, a relation was found between age and multiple follicle growth in a group of 507 women

with spontaneous ovulatory cycles (Beemsterboer *et al.*, 2006). In that study higher mean follicular FSH concentrations were found in women with multiple follicle growth.
In our study there was a tendency to multiple follicle growth in the group of women with variably elevated FSH levels. In these women presumably the follicle cohorts are variable in size from cycle to cycle. The higher FSH levels in the early follicular phase are likely to rescue more than one follicle from atresia, resulting in multiple follicle growth and ovulation.
Our threshold study demonstrated that although follicles have a slightly higher FSH threshold, the actual FSH levels in the early follicular phase are much higher due to decreased inhibition of ovarian hormonal feedback mechanisms. This unopposed overshoot of FSH may stimulate more than one follicle to develop if present.

Clinical consequences of elevated FSH

1. If an elevated day 3 FSH value is found in young women with regular menstrual cycles in the routine fertility work-up, one should realize that the majority of cases will have some kind of limited ovarian reserve. However, this should not result in a negative advice to carry on with fertility treatment (van Rooij *et al.*, 2004). Studies of younger women with elevated FSH levels found no differences in on-going pregnancy and take-home baby rates (Esposito *et al.*, 2002; van Montfrans *et al.*, 2000) and from a systematic review it was concluded that basal FSH in regularly cycling women is not a good predictor for non-pregnancy (Broekmans *et al.*, 2006). Therefore, we should question the value of routinely screening for elevated basal FSH in counselling the subfertility patient.

2. Clinicians should always keep in mind the differential diagnosis of elevated FSH and realize that elevated FSH levels do not necessarily imply a decreased primordial follicle pool.

* As described in chapter 5 the possibility of technical errors in the immunoassay, such as heterophilic antibodies must be considered. This is suspected sometimes with extremely high FSH levels and normal LH values, but can also be the case in moderately elevated FSH levels. The laboratory can perform serial dilution tests and PEG precipitation, cross-check with an alternative assay system, or measure additional hormones (inhibin B, AMH).
* In mothers of familial dizygotic twins, higher FSH levels are associated with an increased secretory drive of FSH.

* The presence of the frequently occurring variant of the FSH receptor gene N680S can also be a cause of relatively higher FSH levels in regular menstrual cycles. This receptor variant seems to be less sensitive to FSH and is overrepresented among patients with subfertility who have elevated FSH levels (chapter 6).

3. Ovarian biopsies to estimate the number of remaining follicles in the ovary should not be taken. The unequal distribution of follicles across the ovarian cortex results in an enormous interbiopsy variation of numbers of follicles per mm^2 surface area (chapter 3).

4. It should be questioned if in IVF stimulation protocols a higher FSH dose might be beneficial in patients with elevated FSH levels, although the FSH threshold is slightly higher (chapter 4). In IVF stimulation, like in the natural cycle in women with elevated FSH levels, the FSH threshold is largely surpassed already. We found in women with elevated FSH levels the FSH threshold to be higher (6.75 IU/l) compared to controls (4.65 IU/l), but in standard IVF protocols FSH levels of 15.8 IU/l on day 6 are reached (Smitz *et al.*, 2007).

Future perspectives and research

The results of the studies presented in this thesis indicate that more research needs to be done to further understand the significance of elevated FSH in regular cycling women. It also directs us to other fields of research related to ovarian physiology and ovarian responsiveness to gonadotrophins.

Our threshold model could be used to study:
1. the relation between the FSH threshold and FSH receptor type.
2. the relation between the FSH threshold (which was higher in patients with elevated FSH levels) and other factors which could possibly bring the threshold back to the “normal” threshold.

In the hypothalamic-pituitary axis could be further studied:
1. the relation between GnSIF and aging.
2. the relation between GnSIF and the dynamics of the LH surge in older women (to find out why the maximal follicle diameter before ovulation is smaller).

The relation between the FSH receptor type and FSH levels could be further studied:

1. to investigate whether patients with the (normal sensitive) Asn/Asn receptor and elevated FSH levels are closer to imminent ovarian failure than patients with the Ser/Ser variant.
2. to investigate the contribution of FSH receptor polymorphisms in IVF stimulation in relation to gonadotrophin stimulation and in terms of pregnancy outcome.

A randomized controlled trial can be constructed:
1. to investigate if IVF results improve in a cycle with normal day 3 FSH.

In chapter 2 we described the correlation between inhibin B in the luteal phase of the preceding cycle and day 3 FSH levels. Others described the relation between inhibin B and follicle cohort size (Seifer *et al.*, 1997; Bancsi *et al.*, 2002). Since the intercycle variation of day 3 FSH is high and probably related to differences in follicle cohort size from cycle to cycle, it would be useful to study prospectively the differences in response in cycles with low and high day 3 FSH levels. This should be done with a standard short stimulation protocol because stimulation of the available cohort in that very month is the intention.

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Summary

This thesis is focused on the background of elevated levels of FSH in the early follicular phase of women with regular menstrual cycles. In the introduction (*chapter 1*) we describe the characteristics of female reproductive aging which is directed by ovarian aging. We review the endocrine aspects of female reproductive aging in particular the elevation of FSH and in a later stage elevation of LH, and the changes in steroids and inhibins. Furthermore the changes in cycle length and follicle growth are discussed, as well as other substances which might be related to ovarian aging like activins, GnSIF and AMH. Other factors related to elevation of early follicular phase FSH, but not to limited ovarian reserve are also discussed.
The aims of the thesis were:

1. To gain more insight into endocrine and ultrasound events in relatively younger women with a history of elevated early follicular phase FSH levels.
2. To evaluate the neuroendocrine mechanisms responsible for elevated FSH.
3. To study the issue of ovarian reserve estimation by taking ovarian biopsies.
4. To evaluate the ovarian FSH threshold in women with elevated FSH and a regular menstrual cycle.
5. To study the distribution of FSH receptor variants in subfertile women with elevated basal FSH levels and a regular menstrual cycle.

Chapter 2 presents the endocrine and ultrasound monitoring study we performed in 22 patients with a history of elevated day 3 FSH levels and 16 controls. Eleven patients showed elevated basal FSH levels in the study cycle ("High, High"; H,H group), whereas eleven had normalized basal FSH levels ("High, Low"; H,L group). AMH was lower in both patient groups compared to controls. In the H,H group, FSH was higher in all phases of the cycle and both inhibin A and inhibin B were lower during the early follicular phase. In the H,L group, FSH was also higher in the early follicular phase and late luteal phase, and inhibin A was higher in the peri-ovulatory phase.
"Normalization" of day 3 FSH in women with previously elevated FSH was associated with inhibin B levels that became normal in the mid- and late luteal phase of the preceding cycle compared to the lower inhibin B levels when day 3 FSH values remain elevated.
The persistently low AMH levels in combination with constantly and intermittently elevated day 3 FSH levels indicate that these younger patients have diminished ovarian reserve. The endocrine cycle profile in patients with consistently elevated basal FSH resembles that of published data from older women. However, patients who present with elevated early follicular phase FSH but normal FSH in the subsequent cycle are

characterized by normalization of inhibin B in the preceding luteal phase, indicating a temporary increase of the available cohort. Peri-ovulatory inhibin A hypersecretion in the subsequent cycle could thus be a result of multiple follicle growth.

Chapter 3 describes the characteristics of episodic secretion of FSH and LH on day 3 of the menstrual cycle in a group of 13 women with elevated FSH levels (> 10 IU/l) and 16 controls. The pituitary response to gonadotrophin-releasing hormone (GnRH) was also measured. The LH and FSH pulse frequency did not differ between the groups. The FSH and LH pulse amplitudes were increased in the elevated FSH group, as well as the LH and FSH response to GnRH. Oestradiol was not different between the groups, but both inhibin A and inhibin B were lower in the patients with elevated FSH levels. We concluded that in these women the pituitary is more sensitive to GnRH.

In *chapter 4*, we studied the issue of ovarian reserve estimation by taking ovarian biopsies. In the evaluation of ovarian reserve, a test to estimate the number of follicles in the ovary has been long searched for. We examined the feasibility of ovarian biopsy for this purpose. We investigated whether any biopsy regimen is representative of the follicular reserve in a human ovary. Three whole ovaries, removed from patients of reproductive age during operations not involving ovarian pathology, were utilized to count the number and type of follicles found in multiple biopsies of 2 and 5 mm and in the whole ovary. Representative results taking into account the total number of follicles found in the whole ovary showed that predicted values based on biopsies were extremely varied. We concluded that due to the huge variation in the distribution of follicles across the surface of the ovary, there is no place for this procedure in clinical evaluation of reproductive aging in the individual patient.

In *chapter 5* the FSH threshold for monofollicular growth in patients with elevated early follicular phase FSH levels was evaluated. The sensitivity of follicles for FSH can be expressed by the FSH threshold. In six patients and thirteen controls the FSH threshold was determined with GnRH agonist desensitization and an ultra-low-dose step-up protocol. The FSH threshold in the patient group with elevated basal FSH levels was 6.75 IU/l and significantly higher than the FSH thresholds of the controls (4.65 IU/l). The FSH screening value on day 3 was 12 IU/l in the patient group and 5.0 IU/l in the controls. In the control group the basal FSH levels correlated with the FSH threshold levels ($r = 0.8$), but in the patients with elevated basal FSH this correlation was absent. In women with elevated early follicular phase FSH levels the FSH threshold is higher but not as high as their basal FSH levels. We believe that intraovarian factors are responsible for the higher FSH threshold. Basal FSH overshoots the threshold, probably because of the limited feedback of the ovary.

Chapter 6 describes three cases in which elevated early follicular phase levels were falsely elevated due to interference in the immunometricassay. If falsely elevated FSH levels are suspected the laboratory can perform serial dilution tests and PEG precipitation, cross-check with an alternative assay system, or measure additional hormones (LH, inhibin B, AMH).

In *chapter 7* the distribution of a polymorphic variant of the FSH receptor (N680S), which is associated with higher FSH levels in the early follicular phase and an increased FSH requirement to obtain follicular response in IVF patients, was described in a group of regularly menstruating subfertility patients with elevated basal FSH levels. This receptor variant is thought to be less sensitive to FSH and higher endogenous FSH levels may represent a natural compensation which is needed to enable normal follicle growth. The aim of the study was to test the hypothesis that this receptor isoform occurs more frequently in patients with elevated basal FSH levels, compared to women with normal basal FSH levels. A retrospective cohort study of 38 patients with a regular menstrual cycle and elevated (> 10 IU/l) compared to 40 patients with normal early follicular phase FSH was carried out. DNA was analysed to determine the FSH receptor genotype. The N680S variant on one or both alleles of the FSH receptor gene was significantly more prevalent in patients with elevated FSH. The conclusion of the study was that the N680S receptor variant of the FSH receptor is indeed more frequently found in women with elevated basal FSH levels and that it suggests a higher FSH threshold.

In *chapter 8* the findings of the studies described in this thesis, the clinical consequences and the future perspectives are discussed. The endocrine profile of younger women with elevated early follicular phase FSH levels and regular cycles is comparable to older reproductive aged women, however, when FSH is temporary normalized higher inhibin A levels were observed, which can be related to multiple follicle growth. The elevation of FSH is the result of relaxation of ovarian inhibin restraint of GnRH-independent pituitary FSH secretion, and relaxation of restraint by GnSIF on GnRH-dependent FSH secretion rather than changes in ovarian steroid hormones. The intercycle variability of day 3 FSH levels can be explained by 1) advanced follicular growth due to an earlier start of the growing follicle which results in a shorter cycle length, and 2) variation of cohort size with temporary normalization of the cohort. Multiple follicle growth is related to higher FSH levels as seen in spontaneous dizygotic twinning and familial dizygotic twinning. In our study there was a tendency to multiple follicle growth in the group of women with variable elevated FSH levels.
The clinical consequences of elevated FSH in young women are discussed. Since basal FSH in regularly cycling women is not a good predictor for non-pregnancy,

we should question the value of routinely screening for elevated basal FSH in counselling the subfertility patient. Clinicians should be familiar to the differential diagnosis of elevated FSH: technical errors in the immunoassay, familial dizygotic twinning and the N680S receptor variant of FSH. Follicles are unequally distributed across the ovarian cortex which makes ovarian biopsies to estimate the number of follicles in the ovary unreliable. Although the FSH threshold in women with elevated early follicular phase FSH levels is slightly higher, it should be questioned if in IVF stimulation protocols a higher FSH dose is beneficial.

Samenvatting

Dit proefschrift gaat over de oorzaken van vroeg folliculair verhoogde FSH-spiegels bij vrouwen met een regelmatige cyclus. In de Introductie (*hoofdstuk 1*) wordt de reproductieve veroudering van de vrouw beschreven die veroorzaakt wordt door ovariële veroudering. Er wordt een overzicht gegeven van de literatuur over de endocrinologie van ovariële veroudering, met name de veranderingen bij vrouwen die een verhoogd basaal (vroeg folliculair) FSH hebben. Verder worden de veranderingen in cyclusduur en follikelgroei besproken en andere substanties die gerelateerd zijn aan reproductieve veroudering, zoals activin, GnSIF en anti-müllerian hormoon (AMH). Tenslotte worden ook andere oorzaken van verhoogde FSH-spiegels besproken die niet met verminderde ovariële reserve te maken hebben. Het doel van dit proefschrift is te onderzoeken wat de mechanismen zijn die leiden tot verhoogd FSH, zoals: de feedback, de bijdrage van de hypothalame-hypofysaire as en de gevoeligheid van het ovarium.

Hoofdstuk 2 beschrijft een studie van de endocrinologie en follikelgroei in 22 patiënten met een vroeg folliculair verhoogd FSH in de screening bij het oriënterend fertiliteits onderzoek, en in 16 controle patiënten. Elf van de vrouwen met verhoogd FSH hadden ook in de geanalyseerde cyclus een verhoogd FSH op cyclusdag 3 (“Hoog, Hoog”; H,H groep), maar er waren ook 11 vrouwen, met een verhoogd FSH in de screening, die een normaal FSH hadden (< 10 IU/l) in de geanalyseerde cyclus (“Hoog, Laag”; H,L groep). Het AMH was lager in beide patiëntengroepen vergeleken met de controle groep. In de H,H groep was het FSH hoger in alle fasen van de cyclus, en inhibine A en inhibine B waren lager in de vroeg folliculaire fase. In de H,L groep was het FSH ook hoger in de vroeg folliculaire fase en de laat luteale fase van de cyclus. Inhibine A was in deze groep hoger in de peri-ovulatoire fase.
“Normalisatie” van het derde dags FSH in vrouwen met een eerder verhoogd FSH was geassocieerd met inhibine B-waarden die normaal waren in de luteale fase in de voorgaande cyclus in tegenstelling tot lagere inhibine B-waarden in de voorgaande cyclus wanneer het derde dags FSH hoog bleef.
De lage AMH-waarden in combinatie met constante of variabele hoge FSH-waarden in de vroeg folliculaire fase wijzen op een verminderde ovariële reserve bij deze jongere vrouwen. In cycli met verhoogd FSH in de vroeg folliculaire fase is het endocrinologisch profiel gelijk aan dat van oudere vrouwen in de peri-menopauze. Vrouwen met een normaal FSH die eerder een verhoogd FSH hadden, worden gekarakteriseerd door normaal inhibine B in de voorgaande luteale fase, wat een indicatie kan zijn van een tijdelijk groter follikelcohort. Peri-ovulatoire verhoging van inhibine A zou het resultaat kunnen zijn van multiple follikelgroei.

Hoofdstuk 3 beschrijft de karakteristieken van pulsatiele secretie van FSH en LH op cyclusdag 3 van de menstruele cyclus in een groep van 13 vrouwen met verhoogde

FSH-serum spiegels (> 10 IU/l) en 16 controles. Ook werd de hypofysaire respons op een testdosis gonadoptrophin releasing hormone (GnRH) gemeten. De LH en FSH pulsfrequentie verschilde niet tussen deze groepen. De FSH en LH pulsamplitude was verhoogd in de groep met verhoogd FSH, en ook de respons op de testdosis GnRH. Oestradiol was niet verschillend, maar zowel inhibine A als inhibine B waren lager in de patiënten met verhoogde FSH spiegels. Wij concludeerden dat in deze vrouwen de hypofyse gevoeliger is voor GnRH.

In *hoofdstuk 4* bestudeerden we het probleem van de schatting van ovariële reserve door het nemen van biopten van het ovarium. Er bestaat al langere tijd een behoefte om de ovariële capaciteit op een directere manier te bepalen. Wij bestudeerden de haalbaarheid van het nemen van biopten uit het ovarium voor dit doel. Onderzocht werd of een of meerdere biopten een representatief beeld kunnen geven van de follikelvoorraad in een humaan ovarium. Drie hele ovaria afkomstig van vrouwen in de reproductieve leeftijd, die verwijderd werden tijdens operaties die niet te maken hadden met ovariële pathologie, werden gebruikt om het aantal en type follikels te bepalen. Dit werd gedaan in meerdere biopten van 2 mm doorsnede en een biopt van 5 mm doorsnede en het gehele ovarium. De resultaten van het gehele ovarium kwamen niet overeen met de voorspelde waarden berekend uit de resultaten van de telling van de biopten. Geconcludeerd werd dat door de enorme spreiding in de verdeling van de follikels over het ovariumoppervlak een representatief biopt niet te nemen is het is dus niet zinvol om een ovariumbiopt te nemen in de klinische evaluatie van reproductieve veroudering.

In *hoofdstuk 5* werd de FSH-drempel voor monofolliculaire groei in patiënten met vroeg folliculair verhoogd FSH geëvalueerd. De gevoeligheid van follikels voor FSH kan worden uitgedrukt in de FSH-drempel. Bij 6 patiënten met verhoogde FSH-waarden en 13 controles werd de FSH-drempel bepaald doot een GnRH-agonist desensitisatie en een ulta-low-dose step-up protocol. De FSH drempel in de patiëntengroep met vroeg folliculair verhoogde FSH-waarden was 6.75 IU/l en significant hoger dan de FSH-drempel van de controles (4.65 IU/l). De FSH-waarde in de screening op cyclusdag 3 was 12 IU/l in de patiënten en 5 IU/l in de controles. In de controlegroep correleerde de screeningswaarde goed met de FSH-drempel ($r = 0.8$), maar in de patiëntengroep was er geen correlatie tussen deze twee waarden. In vrouwen met verhoogd FSH in de vroeg-folliculaire fase is de FSH-drempel hoger, maar niet zo hoog als hun basale FSH-waarde. We denken dat intra-ovariële factoren de hogere FSH-drempels in deze patiënten verklaren. Basale FSH-waarden overstijgen de FSH drempel waarschijnlijk door de beperkte ovariële feedback.

In *hoofdstuk 6* worden drie casus beschreven waarbij verhoogde vroeg folliculaire FSH-waarden vals verhoogd bleken te zijn door verstoring in de immunometrische assay. Als er een verdenking is op vals verhoogde FSH-waarden kan het laboratorium seriële verdunningsreeksen of PEG-precipitatie uitvoeren, een andere assay proberen of aanvullende hormoonbepalingen verrichten (LH, inhibin B, AMH).

Hoofdstuk 7 beschrijft de verdeling van een veel voorkomend één-nucleotide polymorfisme op positie 680 van het FSH-receptor gen (N680S) die geassocieerd wordt met hogere FSH-spiegels in de vroeg-folliculaire fase en een verhoogde FSH-stimulatiebehoefte (lage respons) in IVF-patiënten, in een groep van subfertiele vrouwen met regelmatige cyclus en verhoogde basale FSH-waarden. Deze receptorvariant zou minder gevoelig zijn voor FSH, en hogere endogene FSH-spiegels kunnen een natuurlijke compensatie zijn om bij deze -mindere gevoelige- FSH-receptor normale follikelgroei te laten plaatsvinden. Het doel van de studie was om te onderzoeken of deze receptorvariant vaker voorkomt bij vrouwen met verhoogde basale FSH-waarden vergeleken met vrouwen met normale basale FSH-waarden. Er werd een retrospectieve cohortstudie gedaan van 38 patiënten met verhoogd FSH (> 10 IU/l) vergeleken met 40 patiënten met normaal basaal FSH. DNA-analyse werd gedaan om de FSH-receptor te typeren. De N680S variant op één of beide allelen van het FSH-receptor gen was significant vaker aanwezig bij patiënten met verhoogde FSH-spiegels. De conclusie van de studie was dat de N680S variant van de FSH-receptor vaker wordt gevonden bij vrouwen met verhoogde basale FSH-spiegels met een regelmatige cyclus en het suggereert een hogere FSH drempel.

In *hoofdstuk 8* worden de resultaten behandeld van de verrichte studies die in dit proefschrift zijn beschreven, de klinische consequenties ervan en er worden voorstellen gedaan voor toekomstig onderzoek op dit gebied. Het endocrinologisch profiel van jongere vrouwen met vroeg folliculair verhoogde FSH-spiegels en een regelmatige cyclus is vergelijkbaar met dat van oudere vrouwen aan het einde van de reproductieve levensfase. Maar als het basale FSH tijdelijk normaal is, worden hogere inhibine A-spiegels gezien die gerelateerd kunnen zijn aan multiple follikelgroei. De verhoging van FSH is het resultaat van verminderde ovariële inhibine onderdrukking van de GnRH-onafhankelijke hypofysaire FSH-secretie en vermindering van de onderdrukking door GnSIF van de GnRH-afhankelijke FSH-secretie en niet van steroïde hormonen. De intercyclische variabiliteit van dag 3 FSH-spiegels kunnen worden verklaard door a) de vroegere start van de follikelgroei resulterend in een kortere cyclusduur en b) de variatie in cohortgrootte met tijdelijk grotere follikelcohorten. Meervoudige follikelgroei is gerelateerd aan hogere FSH-spiegels zoals ook wordt gezien in moeders van spontane dizygote tweelingen en familiaire dizygote tweelingen. We vonden een tendens naar multiple follikelgroei

in de groep vrouwen met wisselende FSH-spiegels.
De klinische consequenties van verhoogde FSH-spiegels bij jonge vrouwen worden besproken. Aangezien basaal FSH in vrouwen met een regelmatige cyclus geen goede voorspeller is voor "niet zwanger worden" is het de vraag of routinematig bepalen van basaal FSH in subfertiele patiënten zinvol is. Clinici moeten bekend zijn met de differentiële diagnose van verhoogd FSH: technische problemen in de immunoassay, familiaire dizygote tweelingen en de N680S variant van de FSH-receptor. Follikels zijn niet gelijk verdeeld over het oppervlak van het ovarium en ovariumbiopten, om het aantal follikels te bepalen dat over is in het ovarium, zijn niet betrouwbaar. Hoewel de FSH drempel in vrouwen met verhoogde vroeg folliculaire FSH-spiegels hoger is, is verhoging van de FSH-dosis in IVF-stimulatie bij deze vrouwen waarschijnlijk niet zinvol.

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Curriculum Vitae

Corry de Koning (1965) haalde haar VWO diploma op het Oosterlicht College in Utrecht en studeerde Geneeskunde aan de Rijks Universiteit Utrecht. Daarna werkte zij als arts-assistent chirurgie in Portsmouth (Verenigd Koninkrijk) en in het Hofpoort Ziekenhuis in Woerden. Van april 1993 tot mei 1994 was zij fertiliteitsarts in het Medisch Centrum Alkmaar. Daarna begon zij met haar promotieonderzoek bij de afdeling Voortplantingsendocrinologie en vruchtbaarheidsonderzoek van het Vrije Universiteit medisch centrum in Amsterdam (hoofd: prof. dr. J. Schoemaker, begeleider: dr. C.B. Lambalk). Zij deed ook een onderzoeksstage in Leeds (Verenigd Koninkrijk) bij prof. dr. R.G. Gosden. Zij werkte daar aan een studie over ovariële biopten uit humane ovaria.
In 1998 begon zij haar opleiding tot gynaecoloog in het Onze Lieve Vrouwe Gasthuis in Amsterdam (opleider: dr. M.F. Schutte), vanaf 1999 voortgezet in het VU medisch centrum (opleider: prof. dr. H. P. van Geijn), daarna in het Onze Lieve Vrouwe Gasthuis (opleider: prof. dr. J.M.M. van Lith) en het Sint Lucas Andreas Ziekenhuis in Amsterdam (opleider: prof. dr. F. Scheele).
Vanaf november 2004 werkt zij in het Medisch Centrum Alkmaar. In september 2007 begint zij met de subspecialisatie Voortplantingsgeneeskunde in het Academisch Medisch Centrum in Amsterdam (hoofd: prof. dr. F. van der Veen).
Zij is getrouwd met Rogier Bos en moeder van Jacco en Lotte.

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